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PHYSIOLOGICAL AND CLINICAL TESTS OF AUTONOMIC FUNCTION AND AUTONOMIC BALANCE

CHESTER W. DARROW

Institute for Juvenile Research and Department of Physiology, University of Illinois, College of Medicine, Chicago

To attain significance a test of autonomic function must circumvent the mutually antagonistic action of the two branches of the autonomic nervous system so that it may be clear whether an observed peripheral event is due to increase of activity in one branch of the autonomic system or to decrease of activity in the other. There must be no question, for example, whether an observed pupillary dilatation is due to sympathetic excitation or to inhibition of the parasympathetically determined irido-constrictor tone. The problem is literally to determine the weight on either side of a "balance" when that on neither side is known. The mere knowledge that the balance has been upset by a given condition, as afforded by many so-called tests of autonomic function, may be physiologically or clinically of little value except as indication that something has been disturbed. It does not necessarily define the foregoing events in the neural and neurohumoral systems, and in consequence may even be misleading in determining proper corrective procedures. Furthermore, peripheral autonomic events which now may bear one relation and now another to initiating processes in the nervous system need have no consistent relation to those manifestations of nervous system function known as "behavior." This may explain the sterility which, with few exceptions, has beset attempts to correlate measurements of peripheral autonomic changes with human "behavior."

Circumvention of the difficulties in the pathway to interpretation of peripherally observed autonomic effects in terms of events in the neurohumoral system has been attempted in several ways: 1, by recording changes in those exceptional mechanisms having only a single autonomic innervation, e.g., in the nictitating membranes; 2, by elimination of one of the opposing dual innervations surgically, e.g., by cutting the sympathetic supply to the pupils; 3, by assaying *in vitro* or *in vivo* the output of the respective neurohumoral mediators, adrenalin or sympathin, and acetylcholine: 4, by blocking one of the opposed neurohumoral mechanisms pharmacologically, e.g., by administration of atropine or ergotoxin; 5, by deriving the activity of the autonomic system from the respective effects on differentially sensitive autonomic effectors; 6, by recording the electric activity of the respective autonomic nerves. The main methods of autonomic measurement will be considered in the above order.

The interpretation of findings so obtained is further beset by difficulties imposed by homeostasis, since the rôle of the autonomic system is not merely to produce adaptive changes, but to maintain the internal milieu. Medullary, hypothalamic, hypophyseal, aortic and carotid sinus *feed back* "buffer" or "moderator" mechanisms are as important for neurohumoral control as are the

buffers for controlling pH in the blood. So sensitive are they that the intervention incidental to a given test procedure may actually produce results in certain effectors exactly the opposite of the expected peripheral change. Under 7 we shall consider the mechanisms of maintaining "balance" or *dynamic equilibrium.*

The criteria by which we may determine the sympathetic (orthosympathetic) as opposed to the parasympathetic character of a given mechanism are varied and not always consistent with one another. The following will be tentatively accepted as indicators of sympathetic (orthosympathetic) as opposed to parasympathetic activity pending specific exceptions which may later be referred to: 1, thoraco-lumbar as opposed to cranio-sacral innervation; 2, postganglionic as opposed to preganglionic peripheral innervation, and 3, adrenergic as opposed to cholinergic humoral transmission of impulses to the effector. The possible rôles of the ions potassium vs. calcium, catabolic vs. anabolic metabolism, dehydration vs. hydration, etc., may be given weight in specific instances.

I. *Tests based on autonomic mechanisms having a single innervation.* A. *The nictitating membrane.* The most definite indications of normal autonomic function are perhaps obtained in the case of those effectors having an innervation from only one of the opposed branches of the autonomic system. The autonomic innervation of the nictitating membrane of the cat, as shown by Rosenblueth and Bard (1932) is purely sympathetic[1] and, barring certain antagonistic effects from the skeletal external rectus muscle, eliminated by curare or deep anesthesia, it provides an ideal sympathetic indicator. It is pharmacologically adrenergic, being maximally sensitive to excitatory (E) sympathin (Rosenblueth and Cannon, 1932). That the reaction to adrenalin may be enhanced by eserin and decreased by atropine (Rosenblueth, 1932; Secker, 1937) and that the N.M. may react to large doses of acetylcholine in the animal sensitized by denervation (Morrison and Acheson, 1938) does not seriously detract from its usefulness as a sympathetic indicator under normal conditions. It has been utilized as such an indicator of sympathetic activity, among others, by Acheson, Rosenblueth and Partington (1936), Bargeton (1938), Rosenblueth and Schwartz (1935), Brooks (1933), Brown (1934), Gellhorn and Darrow (1939).

As a sympathetic indicator the N.M. may provide an index not only of sympathetic excitation, but also of a decrease or inhibition of sympathetic tone. Such effects from stimulation of the viscera (bladder and rectum) have been reported by Watkins (1938). Gellhorn and Darrow (unpublished) have observed the effect following traction on the intestines. Rosenblueth and Schwartz (1935) show relaxation of the N.M. following vagal (depressor nerve?) stimulation. Relaxation of the N.M. or decrease of response in the presence of a rise in the blood pressure as reported by these latter authors and by Gellhorn, Darrow and Yesinick (1940) and the exaggeration of N.M. response reported by Rosenblueth and Schwartz following vagotomy and carotid sinus denervation does not detract from the value of the N.M. as an indicator of sympathetic function. Rather, such responses provide examples of the application of this mechanism as an indicator of sympathetic regulation by the moderator nerves.

[1] Bucy (1927) raises question as to a sympathetic innervation of N.M. in the rabbit.

B. *Sweat glands.* Another group of mechanisms having a single innervation and widely used as indicators of intact sympathetic nerve supply are the sweat glands. Lesions of the postganglionic nerves to the periphery produce local anhydrosis of the skin which may be registered as an extreme rise in the electrical resistance (Richter, 1927) with no return of spontaneous activity (Tower and Richter, 1932). After preganglionic section in the cat the rise in resistance was less than after postganglionic section, restoration of function being registered electrically in 28 days (Tower and Richter, 1931; see also Hinsey, Phillips and Hare, 1939).

Another test of sweating is provided by applying to the skin bits of filter paper dried after soaking in a solution of cobalt chloride which changes from red to blue when dry (Wetherell, 1905). Graded series of color changes have been obtained by the writer by adding as hygroscopic agent varying percentages of glycerine to the cobalt chloride solution. For photographic mapping of sweat distribution in neurologic cases the method of Minor (1928) has had wide and successful application (Guttman, 1931; List and Peet, 1938–1939; Richter and Woodruff, 1942; Hyndman and Wolkin, 1941a, b). A solution of iodine and castor oil is sprayed over the patient, after which he is dusted with fine starch powder which turns blue-black in the presence of moisture from the sweat glands. Richter and Woodruff (1941–42) have correlated the iodine-starch with the electrical resistance methods of mapping, demonstrating their correspondence.

Sweating and the concomitant (Darrow, 1927, 1932, 1934) electrical changes of the skin, especially of the palms of the hands, is not only dependent upon an intact sympathetic supply (Richter, 1927) but is extremely susceptible to alterations affecting the level of activity of the central nervous system such as narcosis and sleep (Richter, 1926), and attention or alertness (Darrow, review 1936). While it must be conceded that from sleep to alert attention sympathetic tone is altered more or less in parallel with the activity manifested by the sweat glands, it may still be questioned whether measurements of palmar sweating provide an uncomplicated measure of sympathetic activity. In the first place the sweat glands themselves are dependent upon cholinergic transmission at the effector (Dale and Feldberg, 1934) and conditions altering such cholinergic activity, whether by the amount of available acetycholine, the concentration of cholinesterase, or the presence of adrenalin (Billigheimer, 1920; Langley and Uyeno, 1922; Darrow and Gellhorn, 1939; Darrow review, 1937) limit the level of secretory activity and the magnitude of response to stimulation. The apparent adrenergic limitation of sweating at the effector should be taken into consideration (see sec. V) when sweat measurements are the basis for deductions regarding the activity in the autonomic nervous system.

Furthermore, reflex palmar sweating in response to peripheral stimuli is in great part determined by centers located in the forebrain (Langworthy and Richter, 1930; Wang and Lu, 1930; Schwartz, 1936) and ease of elicitation of the response is dependent to a very great extent upon the state of the higher levels of the brain. As pointed out by others and as we have had repeated occasion to observe (Carlson, Gellhorn and Darrow, 1941) light anesthesia, too moderate

to interfere with sympathetic activity as indicated by nictitating membranes or other sympathetic mechanisms, decreases or may eliminate reactions in the footpads of the cat. Palmar or foot pad sweating, most commonly measured as the "galvanic skin reflex," is not, despite its single sympathetic innervation, an uncomplicated indicator of sympathetic function.

C. *Adrenal medulla.* That the adrenal medulla is another example of mechanisms having a purely sympathetic innervation (Elliott, 1912, 1913; Hollinshead, 1936; McFarland and Davenport, 1941) offers better justification for the utilization of circulating adrenalin as an index of sympathetic activity than the fact that it is a "sympathomimetic hormone," for whereas the production of adrenalin is by sympathetic excitation, the action of adrenalin is both excitatory and inhibitory. Aside from the fact 1, that the peripheral transmission of impulses to the adrenal glands is cholinergic and determined by conditions affecting cholinergic activity (Feldberg and Tsudjemura, 1934), and the fact 2, to be considered in more detail later, that the action of inhibitory (I) adrenalin is the inhibition of parasympathetic or cholinergic mechanisms rather than an effect on sympathetic ones, and the fact 3, that adrenalin by sensitizing the moderator nerves (Heymans, 1929) produces a degree of reflex inhibition of general sympathetic activity, circulating adrenalin (plus undifferentiated sympathin) may be considered an index of general sympathetic function. The assay of humoral mediators is considered in section III.

Other mechanisms alleged to have but a single autonomic innervation such as the pilomotors, the spleen and the uterus have been variously used as autonomic indicators. Those subject to adrenergic inhibition such as the uterus are discussed in section III.

II. *Tests based on the elimination of the nerve supply from one of the opposing branches of the autonomic innervation.* This method has had wide application in the delimitation of responses from dually innervated mechanisms, as witness the use of vagotomy in the attempt to reduce the parasympathetic factors in circulatory changes. The method should be confined to acute experiments in order to exclude the possibility of sensitization of the humoral mechanisms whose neural counterpart it is desired to eliminate. Although maximum sensitization occurs only after nerve degeneration, even within the period of an acute experiment there appears the possibility of a degree of sensitization as suggested by the observations of Darrow and Gellhorn (1939) in the case of the pupil.

A. *The pupil.* One of the most useful applications of this method of control is that of cutting the cervical sympathetic supply to the pupil on one side to permit study of reactions determined by a purely parasympathetic innervation. The difference between the reactions of the parasympathetically innervated pupil and those of the dually innervated structure of the opposite side gives indication also of the concomitant sympathetic activity. In corresponding manner the parasympathetic supply from the third nerve has been severed (Anderson, 1904; Ury and Gellhorn, 1939) to provide a pupil having only a sympathetic innervation. This method of experimental control provides striking experimental confirmation of the fact that it is the inhibition of parasympathetic tone rather than sympathetic excitation which is the predominating factor in

the dilatation of the iris of the cat and rabbit following painful stimuli. The sympathetic supply unquestionably also contributes to the pupillary response after emotional stimuli affecting the hypothalamus (Wang, Lu and Lau, 1932; Carlson, Gellhorn and Darrow, 1941; Hodes and Magoun, 1941) and perhaps also when the inhibitory effects of adrenalin have been eliminated (Darrow and Gellhorn, 1939). The neural mechanisms of the response have been worked out by Karplus and Kreidl (1911), Lieben and Kahn (1930), Bain, Irving and McSwiney (1935), Harper, McSwiney and Suffolk (1935), Harper and McSwiney (1937), McSwiney and Suffolk (1938). The evidence that inhibition of parasympathetic tone plays an important, if not a predominating rôle (possibly different in the monkey, (Bender, 1938; Bender and Siegal, 1940) in a response which has often been referred to uncritically as indicating "sympathetic" activity may well lead to a re-examination of the evidence behind similar interpretations in the case of other dually innervated mechanisms.

B. *Blood pressure.* Perhaps nowhere is such a re-examination more appropriate than in the case of blood pressure. Too often it is uncritically assumed that a sympathetic-like rise in blood pressure in the intact animal is an indication of sympathetic activity, and that a parasympathetic-like fall in pressure indicates parasympathetic activity. That the rise may actually be due to decrease of parasympathetic tone and that a fall may be the result of inhibition of sympathetic tone, as for example by action of the carotid sinus, is often disregarded. The same workers sometimes committing this oversight may take great pains at other times to eliminate the ambiguity of blood pressure interpretation by vagotomy, carotid sinus denervation, atropinization, sympathectomy, and so on. The prevailing lack of correspondence between blood pressure changes and changes in valid sympathetic indicators such as the nictitating membranes, as shown by studies of Acheson, Rosenblueth, and Partington (1936), Rosenblueth and Schwartz (1935), and Watkins (1938) among investigators already mentioned in this paper is proof of the ambiguous significance of blood pressure, assuming of course that the sympathetic system discharges as a whole (Cannon, 1915, 1920; Bard, 1928, 1929). Even after hypothalamic stimulation it was shown by Carlson, Gellhorn and Darrow (1941) that some of the sympathetic-like effects commonly associated with rise in blood pressure may be due to inhibition of parasympathetic tone. On the other hand the fact that Bronk, Pitts and Larrabee (1940) have demonstrated an inhibition of sympathetic impulses in the nerve to the heart following increase of blood pressure in the carotid sinus, and the fact that section of the nerves to the carotid sinus and other moderator mechanisms results in a chronic hypertension of varying duration as shown by Heymans and Bouckaert (1931, 1935), Bacq, Brouha and Heymans (1932, 1934), which can be prevented by prior complete sympathectomy (Heymans and Bouckaert, 1935; Grimson, 1940) points to carotid sinus inhibitory control of the sympathetic system as a factor in blood pressure.

Autonomic control of factors in blood pressure such as heart rate and stroke volume, venous and pulmonary return, and vasoconstriction and dilatation should receive separate consideration. Two will be discussed.

C. *Pulse rate.* The indiscriminate use of pulse rate as an index of sympa-

thetic function in the intact animal is perhaps more to be deprecated than the uncritical employment of blood pressure for that purpose. Not only is pulse rate, like blood pressure, a function of the balance or "resultant" of sympathetic and parasympathetic influences (Rosenblueth and Simeone, 1934; Brown and Eccles, 1934 a, b) but by action of the aortic, carotid sinus, and other moderator nerves upon sympathetic and vagus centers, there tends to be a compensatory slowing of the heart when there is a rise in pressure, and an acceleration with a fall. In addition there is the direct effect of pressure within the heart itself. The clinical value of pulse rate as an indicator depends little or not at all upon its rôle as a sympathetic-parasympathetic indicator (Henderson, Haggard and Dolley, 1927). Even as an index of emotional changes it is of less value than blood pressure (Armstrong, 1938), and its physiologic interpretation is questionable (Gantor, 1925; Shock and Schlatter, 1942). Recorded along with simultaneous changes in blood pressure, however, it may provide valuable indication of compensatory activity by the moderator nerves which should be taken into account in the interpretation of an autonomic change. Pulse rate in the chronically denervated heart is of course determined by humoral mechanisms, to be considered later.

D. *Vasomotor activity.* This is a most important variable determining blood pressure, though obviously not the only one. The inference that there has been vasoconstriction when rise in blood pressure is the sole observation is at best a loose manner of speech. And the frequent identification of vasoconstriction with sympathetic activity and of vasodilatation with parasympathetic action is likewise often in disregard of the facts. Not only do vasomotor functions represent in many instances the resultant of a balance between opposed neurohumoral influences, a change in either one of which may alter the vasomotor tone, but the existence of both sympathetic cholinergic vasodilators (Euler and Gaddum, 1931; Bulbring and Burn, 1934, 1935; Sherif, 1935) and adrenergic vasodilators (Rosenblueth and Cannon, 1935; Wyman and Tum Suden, 1936) is always a threat to such interpretation. Sympathetic vasodilatation has long been recognized as a common characteristic of muscle (Hoskins, Gunning and Barry, 1916; Hartman and collaborators, 1928a, b, and Clark, 1934) where it obviously may serve an emergency function. It is particularly marked in the dog and the hare, "animals of the chase" not only in muscle but in other structures (Langley and Dickenson, 1890; Burn, 1938) and is demonstrable without ergotoxin or eserin. Room (1938) indicated that at least in some cases adrenergic dilatation may be confined to the capillaries and constriction to the arteries and arterioles. However, the skin and splanchnic region of most animals including man most frequently and under most conditions manifest sympathetic adrenergic vasoconstriction and cholinergic vasodilatation.

Of great importance is the fact that evidence indicates the presence of sympathetic cholinergic vasodilator activity in many blood vessels where there are no demonstrable parasympathetic nerve connections. It thus becomes possible readily to explain in these structures the vasodilator effects of parasympathetic drugs and to account at the same time for the depressor effects of weak sympa-

thetic nerve stimulation and of small doses of adrenalin. It is probable that only with strong stimulation or with larger doses of adrenalin will the inhibitory action of sympathin I or inhibitory adrenalin become strong enough completely to counteract this sympathetic cholinergic vasodilator action. The depressor action of adrenalin after ergotamine will later receive a similar explanation. Another mechanism of adrenalin depressor effects is of course the adrenalin sensitization of the carotid sinuses.

Tests of vasomotor function have become of very great concern to the physiologist and clinician because of their usefulness in the attack on the problems of hypertension and other neurocirculatory disorders. These are discussed in reviews by Brown (1936) and by Weiss (1939). They are given consideration under the present heading because of the predominantly sympathetic hyperactivity apparently involved, and the use of sympathetic nerve section as a therapeutic procedure.

Tests of sympathetic vasomotor function are urgently needed to determine the probable benefits of sympathectomy. The question is always whether such radical intervention would sufficiently restore normal circulation to be justified. The use of nerve block (White, 1930; Scott and Morton, 1931) to determine the probable effects of nerve section appears logical. The employment of spinal anesthesia and general anesthesia (Scott and Morton, 1930) and sleep-inducing barbiturates (Craig, 1938) to test the effect of temporary elimination of central sources of sympathetic activity also provide a relatively direct approach. The rationale behind those other tests which employ what appear to be active vasodilatation technics is not so obvious, inasmuch as their operation may be quite independent of an effect on the sympathetic system. The use of fever (Brown, 1926; Adson and Brown, 1929; Adson, 1936), placing the hands in hot water (Landis and Gibbin, 1933), and the employment of cholinergic drugs are examples of this approach. In so far as these latter methods are satisfactory tests of the possible benefits of sympathectomy it must be inferred that they operate by effecting a central impairment or reflex inhibition of sympathetic tone, or that they work peripherally by opposing the action of inhibitory sympathin and adrenalin on the cholinergic vasodilators.

A test of sympathetic reactivity which has had wide application in the diagnosis of hypertension is the cold pressor test of Hines and Brown (1932, 1933), White and Gildae (1937). For this test, the patient rests for 30 minutes after which one hand is immersed in ice water for 2 minutes. A rise in pressure of over 22 mm. is considered indicative of a tendency toward hypertension. Recovery of the pre-test level should occur in the normal person within two minutes. Of special value in the diagnosis of hypertension also is the ophthalmoscopic observation of the eyegrounds (Weiss, 1939; Keith, Wagener and Kernohan, 1928; Wagener, 1933; Hallum, 1936; and Hallum and Gibson, 1938). Tests of skin temperature (Craig, Horton and Sheard, 1933), plethysmographic volume and plethysmographic blood flow are of course useful as are also photoelectric plethysmographic technics (Hertzman and Dillon, 1938, 1940) especially when combined with stimulating procedures of the types described above.

III. *Assay of the output of humoral mediators adrenalin or sympathin and acetylcholine.* The evidence that the transmission of nerve impulses to the effector organs depends upon the mediation of the humoral agents acetylcholine and sympathin needs no elaboration (Dale, review, 1935; Cannon and Rosenblueth, 1937; Butt, review, 1937). The assay of the humoral mediators may be accomplished *in vitro* either by chemical tests or by strips of excised sensitized tissue, and it may be accomplished *in vivo* either in the same or in a second animal by registration of effects on denervated sensitized organs. It should perhaps be pointed out that any humorally determined autonomic response in the absence of nerve activity is, in effect, an *in situ* biological assay of humoral mediator.

A. *Assay of acetylcholine.* Space permits only mention of some of the organic preparations which have been employed. Quoting from Cannon and Rosenblueth (1937):

> According to Gaddum (1936) the relative sensitiveness, measured in γ (0.001 mgm.) per liter, of common indicators of acetylcholine, is as follows:
>
> | | |
> |---|---|
> | Leech muscle (isolated and treated with eserine) | 2 |
> | Rabbit auricle (isolated and treated with eserine) | 4 |
> | Frog heart (Straub method) | 10 |
> | Mouse intestine | 10 |
> | Rabbit intestine | 20 |
> | Frog's rectus abdominis (isolated and treated with eserine) | 20 |
> | Cat's denervated gastrocnemius | 100 |
>
> Since none of these organs is strictly specific, the suggestions of Chang and Gaddum (1933) as to ways of distinguishing between their reaction to acetylcholine and to other substances become important.

Other tests may be mentioned, such as the slowing of the denervated heart and the hypotensive effect (blocked by atropine) of acetylcholine on the blood pressure of the eviscerated chloralose cat.

B. *Excitatory and inhibitory sympathin and adrenalin.* The fact that sympathin, mediating the sympathetic effects is of two types, E and I, excitatory and inhibitory (Cannon and Rosenblueth, 1933, 1935, 1937) must be given more than passing attention. The more rarely mentioned evidence that adrenalin itself is also of two types (Bacq, 1933, 1934, 1940; Greer, Pinkston, Baxter and Brannon, 1938) one of which is primarily excitatory and the other of which is both excitatory and inhibitory is of no less importance.[2]

It has been pointed out by Bacq (1934) that the two types of sympathin, E and I, correspond in their effects to those of an undifferentiated adrenalin and those of an adrenaline, "nor adrenalin," partially oxidized (Bacq, 1935) which has thus been deprived of its inhibitory action.

Even more significant is the usually unmentioned fact that *the inhibitory action of either inhibitory sympathin, I, or of inhibitory adrenalin appears only in structures having a parasympathetic (cholinergic) nerve supply, or those in which, in the absence of a demonstrated parasympathetic supply, the presence of cholinergically*

[2] For simplicity the excitatory and inhibitory effects of adrenalin will here be referred to as "E" and "I," respectively, analogous to Cannon and Bacq's designation of the different types of sympathin.

activated effectors is indicated by a sensitivity to parasympathetic drugs. For example, the contractions of the nerveless amnion of the fowl are inhibited by adrenalin (Langley, 1905; Bauer, 1928) but the amnion notwithstanding its absence of nerve supply is a cholinergically activated mechanism (Bauer, 1928). Even the cholinergic "pseudomotor" contractions of denervated skeletal muscle, most familiar in the Sherrington contraction, are blocked by adrenalin as shown by Gasser and Dale (1926), Dale and Gaddum (1930), Hinsey and Gasser (1930), Bulbring and Burn (1936), and Bender (1938). It should be emphasized that there is no clear evidence of an inhibitory action of adrenalin on purely *adrenergic* structures, except secondarily by way of effects of adrenalin on the cholinergic sympathetic ganglia (Marazzi, 1939) or by way of the carotid sinus and other moderator nerves (Heymans, 1929; Gellhorn, Darrow and Yesinick, 1939; Bronk, Pitts and Larrabee, 1940). Recognition that the antagonistic or inhibitory effects of inhibitory sympathin or adrenalin are functionally inhibitory of cholinergic mechanisms[3] (regardless of what the chemical, permeability, or other mechanisms of that antogonism may be) permits some simplification of the main facts of neurohumeral transmission and of pharmacologic action.

The reaction of the *denervation sensitized nictitating membrane* is that of a *relatively* pure adrenergically excitable structure. It is a sensitive indicator of excitatory (E) sympathin (Hampel, 1935; Liu and Rosenblueth, 1935; Simeone, 1937). The N. M. is little or not at all affected by inhibitory (I) sympathin or the inhibitory action of adrenalin. Extensive utilization of the chronically denervated nictitating membrane for the *in vivo* assay of excitatory sympathin and adrenalin by such workers as McGoun and Ranson (1937), Cattell and Wolff (1934), Rosenblueth and Morrison (1934), Partington (1936), Liu (1935), and Bender and Siegel (1940) bear testimony to its value.

Differentiation of excitatory (E) from inhibitory (I) sympathin was early provided by the differential reaction of the *non-pregnant uterus* of the cat and the *nictitating membranes.* An amount of excitatory sympathin from stimulation of hepatic nerves, which was sufficient to contract the nictitating membrane, had relatively little inhibiting effect on the non-pregnant uterus of the cat (Cannon and Rosenblueth, 1933), whereas inhibitory sympathin from stimulating inhibited structures of the gastrointestinal tract produced a marked relaxation of the uterus (see end of section) but relatively little contraction of the nictitating membrane.

Further differentiation of excitatory from inhibitory sympathin was provided by the nictitating membrane in combination with the response of the pupil. Cannon and Rosenblueth (1935) showed that an amount of excitatory (E) sympathin sufficient to produce a measured contraction of the nictitating membrane occasioned little dilatation of the pupil, although an amount of inhibitory (E plus I) sympathin sufficient to produce the same contraction of the nictitating membrane produced a large dilatation of the pupil. Only after cutting the constrictor fibers of the iris could Cannon and Rosenblueth (1935) obtain appre-

[3] Necheles and Neuwelt (1938) have demonstrated an antagonism between pituitrin and acetylcholin.

ciable pupillary dilatation with their sympathin E. They inferred from this that sympathin E must somehow stimulate the cholinergic constrictor mechanism. However, the evidence that inhibitory sympathin or inhibitory adrenalin relaxes the cholinergic constrictor mechanism of the iris, thus facilitating dilatation, as shown by Joseph (1916), Miller (1926), Poos (1927), Yonkman (1930), and Shackler, Christiansen and Schlossman (1937), offers another if not a better explanation. It is apparent that combined inhibitory and excitatory sympathin may work synergistically by relaxing the constrictor fibers while simultaneously contracting the dilator fibers to produce a greater dilatation than would be possible by excitation alone. Obviously the operation of the humoral mediators E and I adrenalin or sympathin on the pupil parallels the action of nerve stimulation where there may also be synergistic action of sympathetic excitation and inhibition of parasympathetic tone.

When both sympathetic and parasympathetic nerve supplies have been removed the pupil in the eserinized animal provides a valuable indicator of the synergistic combined effects of excitatory and inhibitory adrenalin. Bender and Weinstein (1940) have employed such a denervated iris as an adrenergic indicator along with the denervated facial musculature as a cholinergic indicator.

The gastrointestinal tract, and particularly the musculature of the large and small intestine, have provided some of the earliest and most used tests for the assay of the inhibitory action of adrenalin and sympathin. The fact is again not without significance that motility and tone are here parasympathetically or cholinergically maintained. Indeed, the intestine is itself often used as a test indicator for acetylcholine. Reflex inhibition of these structures is exemplified in the studies of King (1924), Pearcy and Van Liere (1926), Loew and Patterson (1935), and Youmans and Meek (1937). That these inhibitory effects of nerve stimulation are neurologically sympathetic is inferred from the fact that they may be abolished by splanchnicotomy and duplicated by adrenalin. Youmans, Meek and Herrin (1938) employ both innervated and denervated Thierry fistulae in the same dog for the simultaneous testing of both the neurally and the humorally transmitted effects. That the inhibitory effects of nerve stimulation may be mediated in the gastrointestinal tract by an inhibitory sympathin indistinguishable from adrenalin is suggested by the observations of Youmans (1938), Youmans, Aumann and Haney (1939).

The rate of the denervated heart has also been used as a differential indicator for the humoral effects (Cannon and Uridil, 1921; Cannon, Lewis and Britton, 1926; Newton, Zwemer and Cannon, 1931; Rosenblueth and Phillips, 1932; Whitelaw and Snyder, 1934). It is of course sensitive to both cholinergic and adrenergic humoral mediators, but in the absence of eserin or similar drugs the cholinergic effects may be assumed negligible, except as far as it is possible that resident acetylcholine may be subject to adrenergic inhibition. In any case excitatory and inhibitory actions, whether of adrenalin or of sympathin, would work synergistically.

Coagulation time of the blood may also be used as an indicator of adrenergic effects (Cannon, 1929).

Blood sugar is another frequently used indicator in which, barring important corticoadrenal and hypophyseal effects (Soskin, 1941) changes are the resultant of opposed neuro-humoral autonomic influences. The widespread identification of increased blood sugar with sympathicoadrenal function doubtless received its impetus in Cannon's early work with Britton (1925, 1927) and his emphasis on its emergency function in his book on *Bodily changes* (1929). A degree of correspondence between changes in blood sugar and other sympathetic indicators following sympathetic stimulation is shown by Chang (1937) and Bodo and Benaglia (1938). Such correspondence may be eliminated by sympathectomy (Bodo and Benaglia, 1938). Although long used as an indicator of emotion in animals (Cannon and Britton, 1925; Bömer, 1930), only strong emotion is apparently effective in increasing the blood sugar of normal human subjects (Gildae, 1905), and there is general agreement that while elevation of blood sugar may be met with in depressed human subjects, the "emotions" of schizophrenic patients tend to be devoid of hyperglycemic concomitant (Bowman and Kasanin, 1929; McCowan and Quastel, 1931; Whitehorn, 1934; Gildae, Mailhouse and Morris, 1935). The failure to obtain emotional hyperglycemia in these cases may not, as has often happened, be attributed necessarily to a defect in the sympathico-adrenal function, but may, as will appear, be equally well a consequence of an increased vagal activity and insulin secretion.

Dual neuro-humoral control of blood sugar by both sympathico-adrenal and vagal-insulin mechanisms was early shown in morphine hyperglycemia by Houssay and Lewis (1923). They demonstrated that the hyperglycemia could be abolished by splanchnicotomy and that it could then be restored by cutting the vagus. They inferred the antagonistic action of the sympathetic and the vagus. The rôle of the vagal cholinergic innervation of the pancreas (Clark, 1931; La Barre and Vesselovsky, 1933; Babkin, Hebb and Sergeyeva, 1939) as the factor determining the frequent absence of emotional hyperglycemia which has been the concomitant of excitation following adrenalectomy (Britton, 1925; Lumley and Nice, 1930; Harris and Ingle, 1937; McQuarrie, Ziegler, Wangensteen and Dennis, 1939; and Bodo and Benaglia, 1939) has apparently been overlooked until its recent demonstration as a general principle by Gellhorn, Feldman and Cortell. Under a wide range of conditions such as anoxia and convulsant drugs (1940), fever (1941), sham rage, and hypothalamic stimulation (1941), and heat and cold (1941) they demonstrate that after adreno-demedullation there is a consistent tendency for such conditions to produce a hypoglycemia which is reversed if there is subsequently a subdiaphragmatic section of the vagus to eliminate the parasympathetic innervation of the pancreas. Thus blood sugar is added to the list of indicators which must be interpreted as the resultant of a balance of opposed autonomic influences.

Employing the hypophysectomized-adreno-demedullated rat as an extremely sensitive indicator, Gellhorn, Feldman and Allen (1941a, b) demonstrated an abnormally high insulin content in the blood of emotionally disturbed schizophrenic patients. This probably accounts in part at least for the absence of emotional hyperglycemia in the several studies of schizophrenic patients pre-

viously cited. Related and superficially conflicting evidence will be considered later in its relation to the problem of "autonomic balance" (see section VII).

The non-pregnant uterus of the cat, as previously noted, is another of the earlier used indicators of adrenergic inhibitory effects. This organ is unusual in that it is one of the few among inhibited structures having definite sympathetic innervation, but, at least until recently, no established parasympathetic, pelvic nerve innervation (Reynolds, 1939). However, Sheehan and Labate, according to Sheehan (1941) have now "established beyond question the presence of a parasympathetic (sacral) outflow." The important consideration is that despite its sympathetic innervation the uterus is also cholinergically activated (Reynolds, 1939). Sherif (1935) demonstrated acetylcholine secretion by hypogastric (sympathetic) stimulation in the dog.

The effects of adrenalin and sympathetic stimulation is to produce relaxation of the non-pregnant uterus of the cat as contrasted with contraction when the animal is pregnant. This action of adrenalin in the cat is perhaps not surprising in view of the prevalence of other adrenalinergic manifestations in this animal. Van Dyke and Gustavson (1929) and Robson and Schild (1938a, b) showed that the change is dependent in the cat on the secretion of the corpus luteum and may be found in pregnancy, pseudo-pregnancy, or after progesterone (see Kennard, 1937). That the effects are mediated by an altered response to the inhibitory factor in ordinary (E + I) adrenalin and sympathin is indicated by the absence of appreciable relaxation effect by either non-inhibitory sympathin from hepatic nerve stimulation (Cannon and Rosenblueth, 1933) or by non-inhibitory norepinephrine (Greer, Pinkston, Baxter and Brannon, 1933). When, however, according to these latter authors, a uterus is found which, for some reason, does not give the usual relaxation in response to ordinary (E + I) epinephrine, that uterus will give a contraction in response to the relatively non-inhibitory norepinephrine. In other words, when inhibition is not too pronounced, contractility may be demonstrated. In view of the apparent relation of adrenergic inhibition to cholinergic function it would seem that acetylcholine may be involved. Demonstration by Reynolds and Foster (1939) of acetylcholine in the pregnant, pseudopregnant and progesterone treated uterus of the rabbit makes this seem possible. A similar reversal of inhibitory response by physostigmine (Agar, 1940) and by the physostigmine-like action of ergotoxine (see section IV) also implicates acetylcholine. The limited available evidence suggests that animals having a low level of acetylcholine or an absence of cholinergic response to estrogens such as the nonpregnant cat (Reynolds and Foster, 1940) and the rat (Astwood, 1940; Holden, 1939) typically show relaxation in response to adrenalin, and that animals on the other hand which assay a higher acetylcholine content of the uterus during estrus or pregnancy such as the rabbit (Reynolds and Foster, 1939a, b), the dog (Sherif, 1935), and possibly by inference, the pregnant cat (?) typically show contraction in response to adrenalin. This suggests that adrenalin-inhibitory effects may become ineffective in the presence of excess acetylcholine, and that in the absence of inhibition the excitatory effects of adrenergic or cholinergic action may be clearly manifest. This is consistent

with observations on the sphincter of the iris by Joseph (1916), Poos (1927), and Yonkman (1930). This is also the implication of the change of adrenergic relaxation to contraction following administration of physostigmine or of physostigmine-like ergotamine.

There is, however, the complicating fact that during pregnancy ergotamine, while itself producing near maximal contraction of the uterus (Agar, 1940), typically reverses the adrenalin response from contraction to relaxation (Dale, 1906; Cushny, 1906, 1910). This phenomenon has contributed no little to the confusion which has prevailed regarding the mechanism of adrenalin inhibition. It is not improbable, however, that notwithstanding the fact that in general blood flow and muscular activity in the uterus vary independently (Robson and Schild, 1938), the greater susceptibility of the pregnant uterus to circulatory embarrassment is an important factor in this reaction. Vasoconstrictor reactions become more pronounced at this time (Reynolds, 1939) and any deficiency of oxygenation results in a marked increase in the tendency toward uterine contraction (Reynolds, 1939) even to the point of abortion. Under such conditions adrenergic inhibition of uterine vasodilatation may decrease blood flow and occasion contraction of the uterine musculature. On the other hand, after ergotamine, the reversal of the vasomotor response to adrenalin (Robson and Schild, 1938) may favor an improvement in circulation and result in relative muscular relaxation. The rôle of ergotamine and ergotoxine in the blocking of inhibitory adrenergic effects will be considered.

IV. *Autonomic tests involving pharmacologic blocking of one or the other branches of the autonomic system.* This type of test has had application clinically as well as physiologically because the reversibility of the effects permits of their application in human subjects.

A. *Ergotamine, ergotoxine.* This drug has enjoyed a wide reputation as a "sympathicolytic" drug by which sympathetic excitatory effects could be blocked pharmacologically. This has been assumed to permit the differentiation of sympathetic excitatory from other autonomic effects. That this interpretation of its action is misleading, that ergotoxin blocks primarily not sympathetic excitatory (E) but sympathetic inhibitory (I) effects (Rothlin, 1929; Thienes, 1929; Issekutz and Lenzinger, 1928), and that this action is in fact not an action on the sympathetic mechanisms but a protection of parasympathetic activity or a "physostigmine-like" action (Loewi and Navratil, 1926; Matthes, 1930; Linegar, 1939) is supported by the following facts:

The primary action of ergotamine is the contraction of smooth muscle, especially of cholinergically (Sherif, 1935) activated smooth muscle such as that of the uterus (Dale, 1906; Sharp, 1911; Agar, 1940, intestine (Rothlin, 1929), excised sphincter iridis (Crouch and Thompson, 1939), stomach (Smith, 1918), and retractor penis (Dale, 1906). In the intact animal it increases intestinal motility (Planelles, 1925), occasions extreme miosis (Dale, 1906; Crouch and Thompson, 1939), lowers blood sugar, but not after pancreatectomy (Shpiner, 1929) and decreases blood pressure (Wright, 1930). Frequently cases of increased blood pressure have been attributed to contraction of muscular organs. Carotid sinus

desensitization by ergotamine (Heymans, Regniers and Bouckaert, 1930; Bacq, Brouha and Heymans, 1930) may also be a factor in cases of increased blood pressure following this drug.

Even where sympathetic excitatory effects have appeared blocked by ergotoxine, the blocking is not impossibly, as in the case of the nictitating membrane (Rosenblueth, 1932; Rosenblueth, Leese and Lambert, 1933) and the pregnant uterus (Agar, 1940), attributable to the accompanying tonic response or contracture. The sensitivity of these sympathetically innervated structures to acetylcholine has been shown in the case of the nictitating membrane by Morrison and Acheson (1938) and in the case of the uterus by Reynolds (1939). As noted in the foregoing, even the sympathetic impulses via the hypogastric nerves to the uterus are mediated by acetylcholine in the dog according to Sherif (1935). The increased peristalsis (Planelles, 1924), increased excitability of the vagus to acetylcholine, the nausea and emesis, and the uterine contractions attending administration of ergotamine, all point to a physostigmine-like action.

It is the inhibitory effects of adrenalin or sympathetic stimulation on cholinergically activated mechanisms which are blocked by ergotoxine. It is, as already noted, the ability of adrenalin or sympathetic stimulation to relax or block the spontaneous activity and tone in the intestine or the nonpregnant uterus of the cat which is prevented by ergotoxine. Even in denervated skeletal muscle adrenalin inhibition of the cholinergic pseudomotor contraction can be blocked by ergotoxine (Hinsey and Gasser, 1928). Also in the nerve-free but acetylcholine rich human placenta (Chang and Gaddum, 1935) adrenalin constriction of the blood vessels is blocked by ergotoxine (Euler, 1938). And even the familiar reversal of vasomotor response to adrenalin and sympathetic stimulation following ergotoxine are accounted for if we accept the evidence (Burn, review, 1939) for sympathetic cholinergic vasodilator fibers. We may assume that these cholinergic vasodilators normally are inhibited by inhibitory sympathetic action or inhibitory adrenalin, resulting thereby in a constriction which is synergic with the reaction of the adrenergic constrictors. Following ergotoxine this inhibition of the vasodilators does not take place, and there is in consequence a decreased pressor, or even a depressor effect. Bulbring and Burn (1935) and Herwick, Linegar and Koppanyi (1939) showed a similar vasomotor reversal after eserine—abolished by atropine. This again suggests that inhibitory adrenergic effects may be blocked or possibly swamped in the presence of sufficient acetylcholine. That ergotamine may likewise potentiate the depressor effects of acetylcholine and that this action may be reversed to a pressor action by atropine is shown by Linegar (1939) and by Herwick, Linegar and Koppanyi (1939). The previously considered (section III) relation of ergotamine to the reversal of adrenergic response in the pregnant and non-pregnant cat is consistent with this point of view.

That ergotamine may have considerable value as a means of testing for the presence of sympathetic inhibitory and adrenalin inhibitory effects on parasympathetic cholinergic functions appears to be indicated.

B. *Atropine.* The employment of atropine to block the action of cholinergic or parasympathetic mechanisms is the most familiar of pharmacologic blocking technics. Two types of measurements have been sought by this means—1, an index of the normally present parasympathetic activity derived from the changes induced when that activity is blocked, and 2, an index of sympathetic function derived from the magnitude of the total residual activity after parasympathetic opposition is eliminated.

Both of these effects are to some extent obscured or damped by the possible effect of the drug upon cholinergic transmission of nerve impulses within the sympathetic ganglia and adrenals, as well as in the central nervous system, and by the possible compensatory action of the carotid sinus and other moderator nerves.

As early as 1870, Schmiedeberg demonstrated that atropine blocked the inhibitory effects of the vagus on the heart. Escudro (1923) and Danielopolu (1926) devised tests of sympatheticotonia and parasympathecotonia based on the effects of atropine in blocking the parasympathetic control of the heart. Fentress and Solomon (1936) have applied the procedure in the study of autonomic function in psychoneurotic patients.

The contrasting effects of atropine, of parasympathicomimetic and sympathicomimetic drugs have been used as autonomic tests by Myerson, Loman and Dameshek (1937) and by Brohoff, Grosse and Kaldenberg (1938) in the study of the synergistic and antagonistic pharmacologic responses of human subjects. In physiological experiments the use of atropine to determine the rôle of cholinergic mechanisms in a given response is, of course, so common as to be almost routine.

V. *Equating of effects in differentially sensitive autonomic indicators.* The classic instance of derivation of autonomic activity from differential effects on peripheral autonomic indicators is Cannon and Rosenblueth's (see p. 9) differentiation of sympathin E from sympathin I on the basis of the differential effects on the iris or uterus and nictitating membrane.

Darrow and Gellhorn (1939) used a somewhat similar equating technic in contrasting the effects of nerve stimulation on a sympathectomized pupil having only a parasympathetic nerve supply with that of the normal pupil of the opposite side having both parasympathetic and sympathetic innervations. From the denervated pupil it was possible to determine the presence of inhibition of parasympathetic tone following stimulation, while from the difference between that and the normal pupil it was possible to infer the presence of sympathetic excitation.

Darling and Darrow (1938) and Darling (1938) attempted a similar derivation from the differential effects of stimulation upon blood pressure and palmar sweating (galvanic) activity. As the method is more recently being applied (Darrow and Solomon, 1939, 1940) the rationale depends upon the assumption *1*, that blood pressure change represents the difference between opposing effects of sympathico-adrenal and parasympathico-cholinergic influences. Galvanic

or sweating activity on the other hand, depends upon both sympathetic nerve conduction and upon cholinergic transmission at the effector (see p. 3). These relationships are symbolized in the equations:

$$\text{B.P.} = S - P$$

$$\text{and Galv.} = S + P$$

where S represents sympathico-adrenal activity, and P represents parasympathico-cholinergic activity.

Given simultaneous galvanic and blood pressure responses to stimulation these simultaneous equations may be solved for either S or P. Or, if desired, the data may be plotted into two-dimensional graphs or "autonomograms." The results of the method indicate simultaneous effects on both sympathetic and parasympathetic activity in the normal individual with sympathetic effects predominating. They indicate, further, the presence of decreased sympathetic responses and a tendency toward the inhibition of parasympathetic tone following stimulation in "resistant" hostile, inhibited, unco-operative psychotic patients. Evidence from the studies of Darrow and Gellhorn (1939a, b, c) that the presence of adrenalin in the circulating blood disposes toward exaggeration of responses of the type involving inhibition of parasympathetic tone, as well as a diminution of sympathetic responses, suggests that adrenalin may be a factor in determining the reaction pattern of the "resistant" psychotic patients. A possible rôle of adrenalin in accounting for the condition of these patients will be considered under "Autonomic balance" (see section VII).

VI. *Electrical recording of autonomic potentials.* Action potentials of the autonomic effectors or of their innervating nerves provide valuable indications of autonomic activity. A familiar example is the electrical recording of sweat gland activity by the use of endosomatic potentials (Tarchanoff, 1890) (See reviews of galvanic response literature, Landis and Dewick 1929; Landis, 1932; Darrow, 1936, 1937). The nictitating membranes and pilomotor muscles have been recorded electrically (Orias, 1932; Rosenblueth, Leese and Lambert, 1933; Rosenblueth, Davis and Rempel, 1936a, b; Eccles and Magladery, 1937a, b). Effects of ovulation have been registered as abdominal or vaginal action potentials by Burr, Hill and Allen (1935); Reboul, Davis and Friedgood (1937), and Bourdillon (1939). Effects of pharmacologic agents on uterine potentials have been studied by Bozler (1938), Morrison (1940) and Balassa and Gurd (1941). Gastrointestinal motility was studied by Bozler (1938, 1939, 1941), and paccinian corpuscle potentials of the mesentary by Gammon and Bronk (1935). Gastric impulses in the vagus are reported by Partridge and Wilson (1933) and vagal (carotid sinus?) effects by Fischer, Gantt and Lowenback (1934). Comparison of electrical responses in various types of smooth muscle such as the nictitating membranes, pilomotors, intestine, bladder, uterus and ureter by Rosenblueth, Leese and Lambert (1933), Lambert and Rosenblueth 1935, and Davis, Rosenblueth and Rempel (1936), and Bozler (1938) indicate that the initial potentials, types I and II of Cannon and Rosenblueth (1937) are associated with excitation, whether in the case of the sympathetic nerves or in the case of the parasympathe-

tic ones. The effects of various pharmacologic agents on the magnitude of these potentials relates to effects on permeability (Rosenblueth and Cannon, 1936). Inhibitory effects are accompanied only by type III delayed and prolonged potentials, identified by Cannon and Rosenblueth with actual contraction. In the salivary glands the possibility of differential negative as opposed to positive potentials, associated respectively with parasympathetic as opposed to sympathetic stimulation, is indicated by Bronk and Gesell (1926). Pharmacologic effects on the potentials of the submaxillary gland were studied by Rosenblueth, Forbes and Lambert (1933).

Effects of adrenalin, atropine and other drugs in damping cholinergic transmission in sympathetic (cholinergic) ganglia, as well as the facilitating effect of moderate concentrations of parasympathetic drugs on the ganglia have been studied electrically by Marazzi (1939a, b). Potentials from several different autonomic nerves as a means toward the solution of problems of autonomic control have been employed by Hinsey and Gasser (1930), Corbin and Hinsey (1935) and Bishop, Heinbecker and O'Leary (1934).

Especially important are the records of sympathetic action potentials and the correlation of these with cardiovascular control from the hypothalamus and carotid sinus as reported by Bronk, Ferguson, Margaria and Solandt (1936), Bronk, Lewy and Larrabee (1936), Pitts, Larrabee and Bronk (1941), and Pitts and Bronk (1942). The rhythmic character of these potentials and their frequency at about that of the cortical potentials (notwithstanding anesthesia) is of particular interest in view of the evidence assembled by Darrow, Jost, Solomon and Mergener (1942) for an association of alpha potentials with cerebral vasoconstrictor tone.

Of great interest also is the recording of potentials from the carotid sinus and the demonstration of specific functional relationships to sympathetic regulation which could have been derived only by electrical methods (Bronk, 1931; Bronk and Stella, 1932, 1935; Fischer and Löwenback, 1934; Bogue and Stella, 1934, 1935; Samaan and Stella, 1935, and Pitts, 1942). The possible applications of such electrical recording technics (Bronk, Pitts and Larrabee, 1940) are as extensive as the ramifications of the autonomic system.

VII. *Autonomic balance—dynamic equilibrium.* Autonomic dysfunction is often times referred to as "autonomic imbalance." This may be begging the question, for obviously in a balanced system abnormal activity would be recognized only if it were sufficient to upset the balance. A dysfunction of one part of the autonomic system, balanced by a compensatory dysfunction of another part of the system might conceivably constitute a double liability, and yet not be demonstrable as an "imbalance." The futility of treating symptoms under such conditions is obvious, and it is likely that many of the baffling problems of autonomic dysfunctions are of this type.

Although the Eppinger and Hess (1909) concept of balance, of sympathicotonia, and of parasympathicotonia has served a useful purpose in directing and stimulating research, the actual clinical and physiological consequences of its application have unquestionably been disappointing. Without detracting from

the importance of the concept in the development of our thinking, it should be pointed out that present day knowledge of the physiological processes involved in maintenance of "balance" dicatates that discussion to be profitable must be concerned but little with "balance" in the abstract and concentrated rather on specific neurophysiological mechanisms of homeostatic control.

Since the discovery by Hering in 1923 of the main mechanisms of autonomic regulation or maintenance of balance and the confirmation by Koch (1923) and by Heymans (1928) our knowledge of carotid sinus, aortic and abdominal vasosensory mechanisms has advanced so rapidly and the specific determinants of sympathicatonic and vagotonic effects have been sufficiently defined that reference to such terms as "autonomic balance," "sympathicotonia," and "parasympathicotonia" without physiologic qualification partakes almost of mysticism. Such terms are too general and too indefinite, though on occasion it is conceded that they may be useful for purposes of classification.

The carotid sinus mechanism, and also the carotid bodies, at the bifurcation of the internal and external carotid arteries, the vasosensory zone located in the arch of the aorta, and the less important, less well localized "vasotatic" receptors in the abdominal region established by Heymans (1929a, b), Heymans, Bouckaert, Farber and Hsu (1936), and Hsu and Chu (1937) supply neurosensory impulses for the reflex control of circulatory and respiratory equilibrium. The carotid sinuses exercise control by maintaining and varying the tonic inhibition over these most essential life-maintaining functions as well as over the more general emergency functions of the sympathetic system. They determine, as it were, the varying *negative bias* required to "modulate" or moderate oscillations in the circulatory system. In general, optimal conditions of oxygenation and blood pressure which favor bodily activity *activate* carotid sinus function also, and the carotid sinus in turn lowers blood pressure to that minimum which is commensurate with the maintenance of its own activity. Conditions of low oxygen, low blood pressure, or excess carbon dioxide (Bielinski and Wierzuchowski, 1939) in the carotid sinus, on the other hand, tend to reduce or *inactivate* carotid sinus inhibitory processes and release the restrained sympathetic mechanisms from tonic inhibition. At the same time tonic parasympathetic impulses from the carotid sinus are reduced. Such conditions tend, like denervation, toward hypertension, and in more extreme conditions they may occasion hyperpnea (Heymans and Bouckaert, 1930a, b; Winder, 1937a, b, 1938; Schmidt, 1932). The terms *activation* and *inactivation* are here employed relative to the carotid sinus because of the current confusion with respect to the word "sensitization" as applied for example to Vercauteren's (1932) demonstration that in the presence of carbon dioxide the added embarrassment of a standard reduction in intrasinusal pressure will produce a larger compensatory hypertensive effect than a similar change in pressure without the carbon dioxide. Since usage has designated this effect a "sensitization" it is pointed out that in this case one agent merely potentiates the *inactivation* produced by the other (Bielinski and Wierzuchinski, 1939). This general interpretation of carotid sinus function is offered with full consideration of the fact that in extremely low pressure, "paradoxical"

reactions with failure of respiration and blood pressure may obscure normal carotid sinus compensations. It is not assumed that these principles apply equally to the chemoreceptors of the carotid bodies, for these apparently operate by a positive rather than by an inhibitory mechanism of control.

Gesell and collaborators have recently called attention to still another buffering action on ganglionic structures. They point out that acetylcholine is rapidly destroyed by cholinesterase in an alkaline medium, but that it is conserved, increasing its duration and intensity of action, in an acid medium. This provides a direct biochemical buffering mechanism in which accumulation of acetylcholine with its facilitation of myoneural and ganglionic transmission parallels carotid sinus disinhibition of sympathetic activity.

Not only do the moderator nerves normally maintain a regulatory inhibitory control over sympathetic activity, actually reducing the magnitude of vasomotor and other reactions to afferent nerve stimulation (see Rosenblueth and Schwartz, 1935, for nictitating membrane, Izquierdo, 1920, for vasomotor effects) but there is evidence likewise of a positive control by way of the parasympathetic system (Hering, 1927; Heymans, 1928, Bernthal and Motley, 1939). Of effects (blocked by atropine) via the vagus on the heart there can be no question, and even increased intestinal motility and tone has been reported following carotid sinus stimulation (Tournade and Malmajac, 1929). Evidence to the contrary (Thomas and Brooks, 1937; Grimson, 1940) that reflex carotid sinus control is completely lost following sympathectomy does not disprove the existence of normal parasympathetic regulation by the carotid sinus, for the reason that carotid sinus function is dependent both upon the presence of adequate internal pressure, and on the presence of sensitizing adrenalin, either or both of which may be eliminated by sympathectomy.

Adrenalin secretion, so often identified as an emergency blood pressure raising device, apparently plays an important, and not always considered, rôle in the carotid sinus limitation and homeostatic regulation of autonomic activity. The presence of circulating adrenalin in the carotid sinus tends to sensitize that mechanism and increase inhibitory effects both on blood pressure and respiration (Heymans, 1929a, b). This was attributed by Stella (1932) to the increase of blood pressure and the effect of adrenalin on the "centers," but Malmajac, Donnet and Desanti (1935a, b), Battencourt (1935) and Chu and Hsu (1938) showed that the presence of adrenalin actually increases the sensitivity of the carotid sinus mechanisms to the existing pressure. Thus may adrenalin occasion a depressor blood pressure reaction independently of its depressor peripheral effects previously considered. Thus adrenalin by increasing carotid sinus inhibition of the sympathetic may exercise an inhibitory control over medulliadrenal secretion. It thereby provides homeostatic limitation of its own output. In like manner may adrenalin limit the reactivity of other sympathetic functions (Darrow and Gellhorn, 1939; Gellhorn, Darrow and Yesinick, 1939).

Conversely, the action of acetylcholine in the carotid sinus may decrease inhibition of sympathetic activity as shown by Heymans, Bouckaert, Farber and Hsu (1935) resulting in hypertension. This may be considered a cholinergic inacti-

vation of the carotid sinus, since the effects are similar in direction if not in degree to those of denervation. The same apparently may be said of the physostigmine-like action of ergotamine in the carotid sinus, which may also result in hypertension according to Bacq, Brouha and Heymans (1932), again probably as an effect of disinhibition of the sympathetic by depression of carotid sinus reflexes, as demonstrated after ergotamine by Heymans, Regniers and Bouckaert (1930). The increased blood pressures observed in human subjects following ergotamine by Freeman and Carmichael (1936) are possibly so accounted for.

Inhibitory control by the carotid sinus apparently extends likewise over the activities of those tonic postural mechanisms which are sometimes associated with sympathetic function. Not only will increase of intra-carotid pressure block respiration (Bouckaert and Heymans, 1930; Schmidt, 1932) but it will halt shivering (Tournade and Malmajac, 1929) decrease action potentials in skeletal muscle (Spychela, 1935) eliminate the knee jerk (Schweitzer and Wright, 1937) reduce other skeletal reflexes (Kaufman, 1938; Koch, 1932) occasion relaxation and sleep (Koch, 1932), halt convulsions and/or induce sleep in previously narcotized animals aroused by metrazol (Gellhorn, Darrow and Yesinick, 1939). Effects such as hyperpnea, convulsions, increased muscle potentials, increased somatic reflexes and convulsions have been observed following decrease of blood pressure by the same authors. Thus conditions of autonomic equilibrium within the carotid sinus may exert a most important direct influence over the activities of the central nervous system.

The most definitely identified clinical effects of carotid sinus dysfunction are probably the syncopal attacks attributed to carotid sinus hyperirritability (Weiss and Baker, 1933a, b). Ferris, Capps and Weiss (1935, 1936) following Weiss and Baker, have subdivided such attacks into *1*, cerebral; *2*, cardiac, and *3*, vasomotor types, depending upon the probable mechanisms of precipitation. In "cerebral" attacks there is apparently a direct action of carotid sinus reflexes upon the brain or its circulation. In the other types the syncope is secondary to the embarrassment of general circulation. Convulsions in these latter cases are consequent to the syncopal attack (Freedberg and Sloan, 1937) and not directly an effect of carotid sinus hyperirritability, although Lennox, Gibbs and Gibbs demonstrate the possibility of attacks without cerebral anoxemia, by direct action of the carotid sinus on the brain. Weiss and Baker (1933) note that in idiopathic epilepsy (150 cases) carotid sinus pressure does not produce seizures. Marinesco and Kraindler (1931a, b) offer further evidence that epilepsy is actually associated not with carotid sinus hypersensitivity but with hyposensitivity and that it may often be a result of failure of that mechanism to protect the brain from mechanical shocks transmitted by the circulation. That a deficiency of the buffering mechanism is an etiologic factor in some cases of epilepsy is indeed not unlikely, but it seems to the author that electroencephalographic evidence suggests that it is the inadequate buffering of the autonomic discharges to the brain, rather than the lack of hydrodynamic control, which is probably most frequently involved.

The complexity of the problem of testing and interpreting the equilibrium

which is the final product of the moderator nerves is further illustrated in certain cases of schizophrenia. Hypersensitivity and over-regulation by the carotid sinus are here possibly indicated. There is a relative absence of sinus arrhythmia (Whitehorn and Richter, 1937) which, according to Regniers (1920), is a sign of carotid sinus activity. Sympathetic functions are depressed (Darrow and Solomon, 1934, 1939, 1940; Gellhorn, 1938). Blood pressure tends to be low (Truman, Hoskins and Sleeper, 1932). Pulse tends to be slow (Hoskins and Welch, 1932). Postural activity may be inhibited as is evident in many of the so-called "withdrawal" symptoms. The vago-insulin secretion is increased (Gellhorn, Feldman and Allen, 1941) and emotional hypoglycemia is the rule (Bowman and Kasanin, 1929; McCowan and Quastel, 1931; Whitehorn, 1934; Gildae, Mailhouse and Morris, 1935). Insulin desensitization with the development of an anti-insulin factor may occur (Meduna and Gerty, 1932), just as insulin hypersensitivity may follow carotid sinus denervation (Casas and Hinsberg, 1932). And a possible mechanism for such a carotid sinus sensitization in resistant hostile unco-operative patients is the secretion of adrenalin suggested by the studies of Darrow and Solomon (1940). This is further supported by the aggravation of schizophrenic symptoms by subcutaneous adrenalin as reported by Lindeman (1935) and the decreased sensitivity to it (Kanner, 1918, and Freeman and Carmichael, 1935) which is a natural consequence of adrenalin adaptation according to Hoskins and Rowley (1915) and Rudolph (1938). Ergotamine which desensitizes the carotid sinus has proved effective as therapy (Baber and Tietz, 1937).

We may seek, then, tests of autonomic balance in terms of specific moderator compensatory mechanisms. Symptoms of imbalance may conceivably arise *1*, from exaggeration or displacement of one type of autonomic activity such that the normal buffering mechanisms are unable to restore equilibrium; *2*, from a deficiency or insensitivity of the moderator mechanisms; *3*, displacement of activity in one part of the autonomic system and an overcompensation by the buffer mechanisms as far as other activities of the organism are concerned; and possibly *4*, from overactive or oversensitive moderator mechanisms. A tabulation of symptoms might not differentiate these conditions, yet treatment would properly differ according to the cause of the dysfunction.

Tests of autonomic balance which have had wide application are in general of two types (A) those which examine the reaction of the organism or of some of its organs to a specific autonomic stimulus, and (B) those which impose a more or less non-specific load on the organism and attempt to measure the efficiency of the equilibrium maintaining machinery under conditions of regulated stress. Such are really tests, not of static "balance" but of dynamic equilibrium. Space permits only the mention of some of the more common tests of these two types.

A. *Tests of reaction to a specific autonomic stimulus.* Among such tests probably should be included the *oculo-cardiac* test (Aschner) in which pressure on the eyeballs elicits slowing of the heart, said to be marked in "vagotonic" individuals. The effects are different from those for carotid sinus pressure (Mandelsturm and Lipschutz, 1932a, b; Regueiers, 1930; Wright, 1932). The oculocardiac effects,

probably primarily vagal, apparently affect pulse more than blood pressure. Carotid sinus pressure causes both vagal stimulation and sympathetic inhibition. Jacobivici and Nitzescu (1929) employ electrical stimulation of the carotid sinus producing effects similar to pressure. The cold-pressor test previously described (p. 7) is probably effective primarily as a means of sympathetic stimulation. That this test may be effective because it provides a harmless though moderately severe pain stimulus has probably been given less than due consideration. Orthostatic tests, with or without a tilting table may be mentioned in this connection because of action on the carotid sinus. Correspondence of findings with those obtained by carotid sinus pressure appear not too good. The use of autonomic drugs by various routes should doubtless also be included in this group of tests. In the case of intravenous injections interpretation is always complicated by the fact that peripheral effects and central and carotid sinus compensatory effects may work in opposition. The compensatory effect is often the important one. Reactions to intravenous adrenalin and "wheal" and "flare" reactions to subcutaneous adrenalin may have quite different significance. Tests of reaction to carbon dioxide and to overventilation and attempts to shift the acid-base equilibrium as for example with bicarbonate or ammonium chloride deserve mention in this connection, not only because of the relation of ionic equilibrium to ganglionic and myoneural transmission, but more especially because of the activation by hypocapnia, high pH and calcium of the carotid sinus complex.

It must be emphasized that *a priori* interpretations of such tests involving autonomic stimulation is hazardous. For example, the fact that pressure on the eyes produces marked vagal-like effects in certain individuals does not automatically render this a valid test of "vagotonia." Only demonstration of the correlation of these stimulation effects with results of other valid indicators can be justification for such an interpretation.

B. Tests which attain significance because they impose an effective load on the organism and thus provide a means of gauging the effectiveness of various homeostatic mechanisms in handling the load are almost too non-specific to be included as autonomic tests. We should perhaps mention the Schneider (1920) test of cardiovascular efficiency, with its regulated mounting and demounting of a standard stairstep. Tests in which the ability of the organism to cope with a low oxygen pressure and acapnia also deserve mention. Apparently it makes little difference whether the subject ascends high altitudes by breathing a low percentage of oxygen from a Douglas bag, by being subjected to reduced pressure in a tank, or by boarding a plane. This adds to the facility and convenience of making such studies. A third class of tests worthy of special mention are the so-called "neurosis" producing tests which permit study of equilibrium maintaining mechanisms which may be upset in emotion.

Such tests tend to go afield from the category of strictly autonomic tests. Metabolic activity, brain function, electroencephalographic activity, electrocardiographic activity, biochemistry and other functions without number may be and are on occasion properly measured in pursuit of information regarding autonomic functions. The only limitation is what can be measured and what is the aspect of autonomic function under consideration.

Batteries of autonomic tests have been employed for the study of autonomic function and balance with a view toward determining individual differences and to throw light on the rôle of the autonomic system in the broader clinical adaptive and psychological relations. The clinical-pathological studies of Peterson and collaborators (1930–1938) provide an exploration in this field with clues to possible relationships which should be mentioned. The pharmacologic studies of Meyerson, Loman, Dameshek et al. also provide a systematic approach. The correlational studies employing factorial analysis by Darling and by Wenger (1941) suggest possibilities for the application of modern mathematical technics to the solution of autonomic problems where results from batteries of autonomic tests are available. The latter method should reveal any tendency for autonomic test results to be grouped according to any principle. For example, if sympathetic and parasympathetic activities play different and determining rôles these should be derivable by such factorial analyses as indeed they were in the results of Darling. Factorial analysis, it should be pointed out, may reveal relationships which otherwise might escape attention. They cannot, however, impart autonomic significance to tests which are not themselves valid according to acceptable physiologic criteria. Such correlational procedures should nevertheless provide tests for the validity of accepted test technics and assist in the identification of still undiscovered autonomic indicators.

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MUSCULAR DISORDERS ASSOCIATED WITH DEFICIENCY OF VITAMIN E

ALWIN M. PAPPENHEIMER

Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, N. Y.

It has become increasingly obvious from the work of the past few years that the substances designated by the term "vitamin E" play an indispensable part in the metabolism of skeletal muscle. It is the purpose of this review to assemble the evidence in support of this statement.

The first hint that the effects of vitamin E deficiency were not restricted to the reproductive sphere is to be found in the observation of Evans and Burr (1) in 1928 that the offspring of mother rats, partially deprived of vitamin E, tend to become paralyzed toward the end of the nursing period. We may quote from their description of the clinical picture. "A day or two before the weaning period (21st day of life), it is frequently noted that the young in these litters have begun to have difficulty in regaining their limbs when placed on their backs. The disability increases. By the 21st day of life, about three-fourths of such young are paralyzed in part of the musculatures of the body wall and the posterior extremities. The disease increases in severity during the ensuing 4 or 5 days and by the 25th day of life, practically all animals destined to develop the disease will exhibit it, though occasional instances of a still later development have been encountered." . . . "The suddenness of onset is remarkable. One encounters seriously affected animals known to have been normal the previous day" . . . "Less than 20 per cent recovered, and some of these were left with residual paralysis."

"Complete protection was obtained when wheat germ oil or concentrates (non-saponifiable fraction, freed of steroids) was administered to the young." Although vitamin E had not at that time been chemically identified, the evidence pointed to a "complicity of vitamin E in the production of this disorder."

Evans and Burr did not themselves investigate the underlying pathology but placed material at the disposal of Lipschütz (2) who, eight years later, published a paper describing widespread degenerative changes in the central nervous system in these rats. Curiously, the patent, grossly visible alterations of the skeletal muscles completely escaped his observation, and it was not until Olcott's (3) paper in 1938 that the paralysis of suckling rats was recognized as being due to extensive necrosis of the voluntary muscles. Olcott could find no lesions in the central nervous system.

In the meantime, the production of generalized muscular lesions by dietary means had been achieved by other workers in different species. Goettsch and Pappenheimer (4) in 1931 described profound alterations of the skeletal muscles in rabbits and guinea pigs maintained upon a scorbutic diet, supplemented by adequate amounts of orange or tomato juice, and treated with ethereal ferric chloride to destroy the vitamin E (Waddell and Steenbock, 5).

They proposed for this disease the now generally accepted term "nutritional muscular dystrophy." Other species have been found to be susceptible to a similar disorder; sheep and goats (Madsen, McCay and Maynard, 6), ducks (Pappenheimer and Goettsch, 7), the tree kangaroo (Goss, 8), mice (Pappenheimer, 9), and hamsters (10). Symptoms suggesting muscle dystrophy have been noted by Anderson, Elvehjem and Gonce (11) in pups bred of vitamin E deficient mothers, but no studies on the pathology have yet been reported. Dogs with chronic biliary fistulae have been found by Brinkhous and Warner (12) to develop muscle lesions, and these have been ascribed to failure of absorption of vitamin E when bile is excluded from the intestine.

Chicks only exceptionally develop muscle lesions on an E deficient diet (Pappenheimer, Goettsch and Jungherr, 13; Glavind, 14).

Abundant evidence has accumulated to prove that this selective necrosis of the muscle fibers results from a deficiency of vitamin E in the diet. It has been shown that vitamin E in the form of natural or synthetic alpha-tocopherol prevents or cures the disease in rabbits and guinea pigs (Shimotori, Emerson and Evans, 15; Mackenzie and McCollum, 16; Eppstein and Morgulis, 17). The minimal requirement of rabbits for dl-alpha-tocopherol acetate has been estimated by Eppstein and Morgulis on the basis of curative experiments at about 0.32 mgm. per kilo per day, somewhat less than the figure of 0.6 to 1.06 mgm. given by Mackenzie, Levine and McCollum (18).

The paralysis of suckling rats derived from vitamin E deficient mothers can also be prevented by the administration of natural or synthetic alpha-tocopherol, 0.5 mgm. fed on the 15th to the 17th day affording protection to at least 85 per cent of the young (19, 20). The nutritional myopathy of ducklings is prevented by daily doses of 2 to 4 mgm. of synthetic dl-alpha-tocopherol (21).

Two factors have been much discussed as to their importance in the production of experimental muscular atrophy.

The first is whether cod liver oil in the diet of herbivorae exerts a direct toxic effect upon the skeletal muscles or whether it favors the dystrophic change by destroying, through its rancidity, vitamin E in the intestinal tract. The first point of view was maintained by Madsen, Maynard and McCay (6), and by Davis, Maynard and McCay (22), who clearly demonstrated that the addition of cod liver oil expedites and intensifies the muscle lesions. Nevertheless, the complete omission of cod liver oil from the experimental diet does not entirely eliminate the injury. Goettsch and Pappenheimer (4), Madsen (23) and Mackenzie and McCollum (24) have shown that muscular dystrophy can be produced in guinea pigs with diets containing no cod liver oil. This would seem to exclude toxicity of cod liver oil as a primary factor in the production of the lesions. Mattill (25, 26), demonstrating the vulnerability of vitamin E to oxidation in the presence of unsaturated fatty acids, strongly questions the assumption of a direct toxic action. In a recent paper, Mattill and Golumbic (27) bring additional evidence that the effect of cod liver oil is to favor the destruction of vitamin E through the development of rancidity in the gut.

Another question which may be regarded as settled is the alleged participation

of a water soluble, not further identified member of the vitamin B complex in the deficiency. A dual deficiency was first postulated by Morgulis and Spencer (28). At that time pure preparations of alpha-tocopherol were not available. Further work on the prevention and cure of the disease in both rabbits and guinea pigs has convincingly excluded the complicity of a deficiency of a water soluble B factor in the causation of the lesions.

It was recently reported by Holmes and Pigott (29) that orally administered thiamin chloride was effective in curing the suckling paralysis of rats. The authors appear to have been unfamiliar with the spontaneous regression of the lesions after weaning. Terry (30) has proven the ineffectiveness of vitamin B_1 in the prevention and cure of suckling paralysis.

CHEMICAL STUDIES. 1. *Changes in electrolytes.* Fenn and Goettsch (31) analyzed dystrophic rabbit muscle for K, Na, Cl, Mg and Ca. Potassium and magnesium were found decreased; sodium was increased out of proportion to gain in chloride, from which it was concluded that it entered the cells in exchange for potassium and magnesium. High concentration of Ca and total P were found in muscles showing histologic evidence of calcification of the fibers.

A similar study by Morgulis and Osheroff (32) confirmed the loss of potassium and the increase in the sodium and chloride content. The calcium was also increased. The interpretation of these over-all changes in electrolyte content is difficult because of the complex and varying composition of dystrophic muscles as regards normal, necrotic, and regenerating fibers, wandering cells, interstitial fat and connective tissue, and pathologic calcification of dead fibers.

2. *Phosphorus compounds.* Morgulis and Spencer (33) found a decrease in total and soluble phosphorus and fractions thereof. Goettsch, Lonstein and Hutchinson (34) analyzed normal and dystrophic rabbit muscle for total phospholipid, total acid soluble phosphorus and total inorganic orthophosphate phosphorus. No striking changes were found until the muscle became severely degenerated. No change was detected in the total phospholipid phosphorus. Total acid soluble, and total inorganic phosphorus were decreased except in the presence of calcification, in which case, as might be predicted, the total acid soluble and total inorganic phosphate were increased. The phospho-creatin content of degenerated resting muscle was reduced absolutely, but was unchanged relative to total acid soluble phosphorus.

Lu, Emerson and Evans (35) have also determined various phosphorus fractions of dystrophic rat muscle, as compared with muscle of rats on normal diet. There was a marked increase in total P and total acid soluble P, no change in creatin phosphate, a slight decrease in organic phosphate. The ability of the affected muscle to phosphorylate glycogen was reduced by about 46 per cent as compared with normal muscle.

There is nothing in these studies to indicate that the described changes in phosphorus fractions precede the onset of lesions, nor do they offer any clue as to the intimate nature of the disturbed metabolism.

3. *Lipids.* Morgulis, Wilder, Spencer and Eppstein (36) studied the lipid content of various tissues of rabbits with muscular dystrophy. They found no

changes in the heart and various internal organs, but the skeletal muscles showed a great increase in fat, lipoid P and especially in cholesterol (100 to 350 per cent). The rise in cholesterol preceded that of total lipoid P and fat, and must be regarded as a "specific characteristic" of dystrophic muscle, the proportion of esters to free cholesterol also increasing as the dystrophy progresses. There is an accompanying hypercholesterinemia (33).

4. *Creatin.* A number of workers (37, 33, 38) have demonstrated a reduction in the creatin content of dystrophic muscle of guinea pigs and rabbits, and of old and young rats (39, 40). An increased urinary excretion of creatin in vitamin E deficient rats was reported by Verzár (41), and in rabbits by Morgulis (42). Advantage has been taken of this creatinuria in following the progress of the disease in rabbits; its disappearance under vitamin E therapy has been a valuable test of the efficacy of the treatment (43, 44).

5. *Oxygen consumption.* One of the most interesting and perhaps significant alterations in the biochemistry of dystrophic muscle is the increased O_2 consumption. Victor (45), using a modified Warburg technic with Fenn volumeters, was able to demonstrate an average increase of from 200 to 400 per cent in rate of O_2 consumption per gram per minute when dystrophic rabbit muscle was compared with normal muscle from animals on a stock diet. Madsen (23) repeated these experiments with muscle from both rabbits and guinea pigs, and fully confirmed Victor's observations. Friedman and Mattill (46) likewise found an increased O_2 uptake in dystrophic muscle of rabbits when compared with that of animals on Purina rabbit chow; and a similar increase of more than 40 per cent was obtained in the muscles of 5 months old female rats kept since weaning on E deficient diet. At 13 months, when the rats had severe paralytic symptoms, the difference tended to disappear, and the Q O_2 was hardly above normal. A few preliminary experiments indicated that the administration of 5 mgm. of dl-alpha-tocopherol acetate was followed by a fall in O_2 consumption within 24 to 120 hours.

Houchin (10) has made similar observations in the hamster. An average Q O_2 of 1.70 was obtained in the semitendinosus muscle of normal control animals. The Q O_2 of degenerated muscle was as high as 4.55. After therapeutic doses of alpha-tocopherol, normal values were reached within 30 hours.

A recent preliminary report by Houchin and Mattill (47) adds the very important observation that water soluble alpha-tocopherol phosphate dissolved in Ringer-Locke solution, brings about a reduction of 41.3 per cent and 35.7 per cent respectively in the O_2 consumption of dystrophic rabbit and hamster muscle. They believe that the alpha-tocopherol participates in a biological system, and does not act merely as a chemical antoxidant. The metabolism of normal muscle was not affected by the addition of alpha-tocopherol, so that it may be assumed that it already contains the optimal amount.

All the preceding studies on O_2 consumption have been made on dystrophic muscle in which the interpretation of the results may be complicated by the presence of inflammatory or regenerating cells, or a variable proportion of fibrous or adipose tissue. Usually the metabolism of muscle from animals on a

stock diet of very different composition from the experimental one has been taken as a normal standard of reference. It therefore seemed important to find out whether a rise in the *in vitro* O_2 consumption on vitamin E deficient diet occurred also in the absence of morphologic changes in the muscle, and whether the administration of alpha-tocopherol, without other modification of the diet, would inhibit this rise. The problem has been taken up by Kaunitz and Pappenheimer (48). The skeletal muscle of young rats born of vitamin E deficient mothers has been found to show a significant increase in Q O_2 as compared with litter-mates which had received a single dose of dl-alpha-tocopherol acetate on the 15th day; and this increase occurred not only in dystrophic muscle, but also in the absence of lesions. The pectoralis muscle of chicks on an E deficient diet was also studied. In this species, lesions of the muscles occur only exceptionally and were completely absent in the material examined. The findings were comparable to those of rat muscle.

These observations complement those of previous observers by demonstrating that the increased O_2 consumption reflects an altered metabolism of the muscle tissue itself, and is independent of visible alterations in structure.

6. *Total O_2 consumption of intact animals on vitamin E deficient diet.* This has been little studied. Wood and Hines (49) discovered no difference in the cubic centimeter O_2 per M^2 of surface area per hour, between guinea pigs on Goettsch-Pappenheimer dystrophy-producing diet 13, and on a stock diet of grains, alfalfa and lettuce.

Kaunitz and Pappenheimer (48) have followed the total O_2 consumption of rats from the 10th to the 150th day. As controls, in addition to rats on a stock diet (Rockland pellets), litter-mates were given a single dose of 1 mgm. of dl-alpha-tocopherol on the 15th day, completely protecting them from the lactational paralysis. The O_2 consumption measurements were carried out in a closed cuircuit apparatus at 29°C; the animals were maintained in a warm room at about the same temperature. Determinations were made at 1 or 2 day intervals during the early growth period, and weekly thereafter. Results were calculated as cubic centimeters O_2 per kilo per minute, and as cubic centimeters per M^2 surface per minute (Lee formula), both on basis of weight and age. From the time of weaning until the 70th to 80th day, or until the rats had attained a weight of about 100 grams, the oxygen consumption of rats which had received a single protective dose of dl-alpha-tocopherol during the lactational period remained consistently and significantly lower than that of their litter-mate controls. After this time there was no appreciable difference.

During the paralytic period, from the 18th to the 23rd days, the affected rats usually lost weight, probably because of inability to nurse, and their subsequent growth was retarded, as compared with treated litter-mates. To determine whether the subsequent increase in Q O_2 was due to this transient weight loss, rats on a complete stock diet were subjected to partial starvation from the 17th to the 21st day. This resulted in a temporary depression in total O_2 consumption, but this was rapidly ironed out, and after the 21st day there was no significant difference between the starved rats and their litter-mate

controls. It would seem therefore that the effect of the tocopherol in lowering the total oxygen consumption throughout the adolescent period is a specific one, and that transient inanition during lactation *per se* does not influence the subsequent O_2 consumption.

The question may be raised at this point whether the increased metabolism in the vitamin E deficient animals is to be attributed to the altered metabolism of the muscle tissue, or whether it is possibly an indirect effect mediated through the thyroid. The literature bearing upon the condition of the thyroid in vitamin E deficiency is rather conflicting. Singer (50) reported the thyroid to be histologically hyperplastic in female rats which had been on an E deficient diet for 12 to 18 months; and Barrie (51) observed the same thing in the young of vitamin E deficient mothers. Telford, Emerson and Evans (52) were unable to confirm Barrie's findings, and Morgulis (53) states that the relative dry weight of the thyroid in dystrophic animals on a vitamin E diet is unchanged. On the other hand, Biddulph and Meyer (54) found a two-fold increase in the weight of the thyroid in male rats after 6 months on vitamin E deficient diet, as compared with those receiving wheat germ oil or stock diet. The addition of iodine to the E deficient diet restrained the thyroid weight to normal limits. In the female they found no difference. In a second paper (55) hypertrophy of the thyroid is brought into definite relation with the low iodine content of the experimental diet and drinking water. The addition of wheat germ oil to the diet, without iodine supplement, alone reduced the thyroid weight and basal metabolism to normal limits.

It is obvious that the increased O_2 consumption noted by Kaunitz and Pappenheimer during the period from 1 to 3 months cannot be ascribed to increased thyroid activity, since it is inhibited by alpha-tocopherol without other supplement. Moreover, the Hawk-Oser salt mixture in their experimental diet contains abundant iodine. The direct effect of alpha-tocopherol phosphate *in vitro* upon the muscle metabolism, as described by Houchin and Mattill, is further evidence against participation of endocrine glands.

Physiological studies. There have been few studies of muscular functions in vitamin E depleted animals. Victor (45) has determined the chronaxie and rheobase in dystrophic rabbit and duck muscle, and found them both significantly increased. Knowlton and Hines (39) have studied the functional capacity of the gastrocnemii of adult rats on a vitamin E deficient diet. The animals were survivors of offspring of vitamin E depleted mothers. The maximum isometric tension developed by the intact gastrocnemius in response to condensor discharges at 50 per second, was found to be definitely less than that of control animals receiving wheat germ oil. Histological examination of the muscle showed hyaline necrosis in only a few fibers, so that the functional alteration appeared not to be closely correlated with visible changes in structure. In a subsequent paper, Knowlton, Hines and Brinkhous (56) demonstrated that synthetic dl-alpha-tocopherol, like wheat germ oil, favorably affected the muscular strength. Both preventive and curative experiments were made.

Effect of inactivity. Section of the sciatic nerve or of the Achilles tendon in

young rats born of vitamin E depleted mothers has been found to protect the corresponding gastrocnemius from becoming dystrophic, provided the operation be performed before the 17th day (57, 58). The significance of this observation is not clear, but it does suggest that vitamin E is in some way implicated in the contractile phase of muscle metabolism rather than in the resting phase.

SMOOTH MUSCLE. There is little to indicate that vitamin E deficiency has a profound effect upon non-striated muscle. Goettsch and Pappenheimer (4) found the smooth muscle of intestine, bronchi, blood vessels and uterus unaffected in rabbits and guinea pigs. In female rats, however, there does occur, after long-standing vitamin E deficiency, a very striking brown coloration of the uterine musculature, not only in animals which have had repeated fetal resorptions, but in virgin rats as well (59, 60, 61). The pigment accumulates within the muscle fibers and interstitial cells, and may lead to degeneration and fibrous replacement. It does not give an iron-reaction, but hemosiderin does occur in the mucosa and interstitial tissue after repeated resorptions (Hessler, 62). The reactivity of the uterine muscle to various pharmacologic agents—acetyl-choline, pilocarpin, prostigmin, adrenalin, ergotamin—was found by Hessler not to be altered. The source and chemical nature of the pigment have not been satisfactorily determined.

The only animal thus far studied in which smooth muscle appears to be selectively affected is the turkey, in which vitamin E deficiency regularly leads to necrosis of the gizzard musculature, with subsequent replacement fibrosis (63).

NERVOUS SYSTEM. Because of the large array of clinical papers which have appeared during the past two years, dealing with the effects of vitamin E therapy in certain diseases of the central nervous system, it seems desirable to review with some care the experimental work upon which this treatment has been grounded.

We have already referred to the paper of Lipschütz (2), who studied the brains and cords of young rats with suckling paralysis, supplied by Evans and Burr. He described (on the basis of Marchi preparations) degenerative changes in *a*, the crossed and homolateral, descending and ascending vestibular tracts; *b*, the columns of Goll and Burdach; *c*, the tecto-spinal tract. With Nissl preparations, it was possible to detect three or four stages of degeneration of the medio-ventral and lateral anterior horn cells, which alone were affected,—vacuoles, diffuse coloration of the cytoplasm, loss of nucleus and Nissl substance. These changes were accompanied by neuroglia proliferation.

Lipschütz attributed the paralytic symptoms in suckling rats entirely to these neurologic lesions, having failed to recognize the presence of severe changes in the skeletal muscles. These were first described by Olcott in 1938 (3), and later by Pappenheimer (64), Barrie (65), Verzár (41), Demole and Pfalz (66) and by Evans and collaborators (67, 68). Olcott and Pappenheimer were unable to find any changes in the central nervous system.

Undoubtedly the most thorough study of the central nervous system in vitamin E deficiency is that of Einarsen and Ringsted (69) published in 1938. This is invariably taken as supplying the experimental ground-work for the therapeutic use of vitamin E in amyotrophic lateral sclerosis and other chronic

nervous disorders (70–91). Ringsted (92) in 1935 had observed certain clinical abnormalities of gait and behavior in adult rats that had been kept on vitamin E deficient diet for a prolonged period. The animals developed a waddling gait, with inco-ordination and spreading of the hind legs, hypesthesia and hypalgesia, roughening of the pelage and loss of hair. Some of the rats survived for 2 years in spite of the progressive paresis.

The lesions found by Einarsen and Ringsted did not tally exactly with those described by Lipschütz. The first structures to be attacked, according to these authors, were the proximal parts of the posterior roots, and the proprioceptive and possibly also the uncrossed paths in the fasciculus cuneatus and fasciculus gracilis. Later, there appeared increasing degeneration in the motor cells of the anterior horns, to which was attributed the progressive atrophy of the leg muscles. It was doubtful whether the peripheral afferent fibers were affected; changes in the spinal ganglia were relatively slight. In several animals, clinically in the last stages of the disease, partial degeneration of the cortico-spinal tract was noted. No changes were found in the tecto-spinal tracts, nor in the nuclei of the medulla or mesencephalon.

As a rule, the "first and largest site" of the process was in the lumbo-sacral part of the spinal cord. The process gradually extended upward to the thoracic and lower cervical cord as far as the 5th segment. In a few cases the cervical cord seemed most severely affected.

The anatomical picture thus resembled closely a combination of tabes dorsalis and progressive muscular atrophy. In the few cases in which the pyramidal tract was affected, the lesions were regarded by Einarsen and Ringsted as analogous to those of amyotrophic lateral sclerosis. The muscle lesions were interpreted as secondary to the spinal cord changes—a simple, neurogenic atrophy with occasional increase in the sarcolemma nuclei.

Monnier (93) has also described alterations in the central nervous system in adult rats on vitamin E deficient diet. His material consisted of 38 adult rats which had been maintained by Verzár on a diet of starch, casein, lard, salts, cod liver oil and brewer's yeast. Symptoms were observed after 9 to 10 weeks and became progressively more severe. Three of the animals died between the 14th and 23rd months, with a loss of weight of 40 to 70 grams in the final 12 days. In addition to the symptoms described by Einarsen and Ringsted, the rats displayed head tremor, dysmetria, diminution in olfactory and auditory sensation, trophic or vegetative disturbances, exophthalmos, urinary incontinence, alopecia and ulceration.

The skeletal muscles showed segmental necrosis, multiplication of sarcolemma nuclei and invasion of mononuclear elements. These muscular changes were associated with partial demyelinization of the intra-muscular branches of the peripheral nerves, and with slight rarefication of the posterior columns of the cord. Later there occurred marked degeneration of the fasciculi of Goll and hyperchromatosis and sclerosis of anterior horn cells. Contrary to the findings of Einarsen and Ringsted, the pyramidal tracts were never affected.

In this country, similar studies have been made by Gutierrez-Mahoney (94) on rats supplied by Dr. Karl Mason. Young rats with paralysis, offspring of

vitamin E depleted mothers and surviving rats, maintained for over a year on the vitamin E deficient diet and comparable to the adult rats of Einarsen and Ringsted, furnished material for this work.

Emphasis is placed on changes found in the ganglion cells—hyperchromatosis of cytoplasm with loss of Nissl substance, thinning and elongation of cell bodies; or a second type of change characterized by diminution of stainable contents and vacuolization and phagocytosis. Both anterior and posterior horn cells, the nuclei of the medulla, Purkinje cells, the larger and smaller cells of the dorsal ganglia and the cells of the cerebral cortex were affected. Myelin stains disclosed alterations in the sciatic nerves—swelling, fragmentation, ball formation and loss of myelin. The anterior and posterior roots in the cauda equina and long conducting pathways were also involved. Changes were seen in the anterior and lateral tracts, in the posterior columns and to a lesser extent in the cortico-spinal pathways and the corona-radiata and median forebrain bundles. Thus the lesions described by Gutierrez-Mahoney were even more severe and widespread than those noted by Einarsen and Ringsted.

We have listed the various lesions described by these authors in some detail. It is plain that there are wide discrepancies in the findings; indeed, no two investigators agree as to the distribution of the lesions.

Wolf and Pappenheimer (95) have recently reported on the results of their study of this problem. Their material included 19 rats on vitamin E deficient diet, comprising 11 adult males, 4 females killed after periods varying from 180 to 365 days, and 4 young. Control animals on stock diet, on a simplified complete diet, and on vitamin E deficient diet supplemented by wheat germ oil or by alpha-tocopherol were taken for comparison. Through the courteous cooperation of Dr. Karl Mason, rats from his colony exhibiting the characteristic signs of chronic vitamin E deficiency were also placed at their disposal.

Aside from changes caused by technical artefacts, and present with equal frequency in the control material, they found the central nervous system unaffected in vitamin E deficient rats.

Whether this cardinal discrepancy between their work and that of previous observers is attributable to subtle differences in diet, to differences in technical procedure or to differences in interpretation, it is difficult to say. As regards diet, Einarsen and Ringsted's diet V was extremely low in fat; nevertheless they found that the addition of oleic and linoleic acid, in addition to 50 mgm. of peanut oil daily, did not prevent the appearance of the lesions. The diets used by Lipschütz, Monnier and by Gutierrez-Mahoney were practically identical with those in use in this laboratory. It is beyond the scope of this review to discuss the technical pitfalls which may give rise to wrong interpretations in the histologic study of the central nervous system, but it seems probable that the diversity in the distribution and character of the reported lesions may be ascribed to variations in technique. One may also call attention to the lack of adequate control material for comparison. The proper controls in this instance—animals on vitamin E deficient diet supplemented by protective doses of alpha-tocopherol were not studied by the authors cited.

In view of these contradictions, it would seem that the therapeutic use of

vitamin E in amyotrophic lateral sclerosis and other degenerative diseases of the spinal cord rests upon a very insecure experimental basis.

There remains the possibility that the primary effect of the deficiency is upon the motor end plates of the muscles, and here again there is no complete unanimity in the reported findings. Rogers, Pappenheimer and Goettsch (96) were unable to detect visible alterations in the terminal neurites or end plates of dystrophic guinea-pig muscles, and Pappenheimer (9) reported similar negative observations in young rats with severe muscle lesions. Telford (97), on the other hand, found a reduction in the number of end plates in severely damaged muscles, with a return to normal upon recovery. There is at least complete agreement that the necrosis of muscle fibers precedes any loss of neurites, and that the nerve endings may be morphologically normal even when necrosis of the muscle has taken place. The possibility that there may be functional alterations in the absence of demonstrable morphological lesions has not been ruled out.

Lesions of the brain in chicks on vitamin E deficient diet. Under the designation of nutritional encephalomalacia, Pappenheimer and Goettsch (98), in 1931, described extensive ischemic necrosis occurring in chicks on a diet deficient in vitamin E.

The first symptoms indicative of severe cerebral damage appeared between the 20th and 30th day after hatching—coarse tremors, ataxia, head retraction, propulsive, retropulsive or rotational movements, usually ending in death. Some animals recovered and went on to normal development, a phenomenon comparable to the spontaneous regression of the lesions in the suckling paralysis of rats. The pathology is distinctive. The lesions affect the cerebellum, cerebrum and medulla in that order of frequency and are essentially areas, often large and grossly visible, of ischemic necrosis. There is death of ganglion cells and glial elements, hemorrhage, edema and capillary thrombosis. Later, if the chicks survive, reparative changes take place (99).

The disease is preventable by various vegetable oils and by their non-saponifiable fractions after removal of the sterols (100); and by alpha-tocopherol, natural or synthetic, in daily dosage of 0.1 to 0.2 mgm. (101, 13). The disease can be produced on a complete diet in which vitamin E is destroyed by heating with ethereal ferric chloride (102), and it occurs as a spontaneous disease under field conditions (103). Although the brain in vitamin E deficient chicks bears the brunt of the injury, the lesions cannot be interpreted as a specific toxic effect upon nerve tissue, since they are obviously the effect of vascular occlusion. The pathogenesis of the lesions is still obscure, whether due to vascular spasm or to capillary thrombosis, but their infarct-like nature cannot be denied.

The "alimentary exudative diathesis" described by Dam and his co-workers (104), and preventable by large doses of alpha-tocopherol, appears not to be correlated with the encephalomalacic condition. It has occurred very rarely with the standard diet 108 used in this laboratory, and prolonged extraction of the casein with alcohol and the low fat content of the diet seem to be, if not essential, yet favoring factors in its onset (105).

Mason (106) has recently called attention to the frequent occurrence of cu-

taneous and intracerebral hemorrhage in rat embryos of vitamin E deficient mothers. Subcutaneous edema, with or without muscle necrosis, occurs also in new-born mice, born of vitamin E depleted mothers (9). It would seem that in certain species and under certain conditions not yet defined, vitamin E deficiency is attended by circulatory disturbances, and perhaps by alteration in capillary permeability.

General discussion. In concluding our review, we may summarize this analysis of the literature with the generalization that vitamin E plays an essential part in the metabolism of skeletal muscle in all species of mammals that have been investigated and in ducklings. It has, in our opinion, not been satisfactorily shown that vitamin E is essential to the integrity of the nervous system.

There are many interesting and fundamental problems that demand further inquiry. The most pressing, it would seem, is to define in chemical terms the exact rôle that the tocopherols enact in the complex chemism of skeletal muscle. With active, water-soluble preparations now available, progress along this line is inevitable. The observation that immobilization of muscle, whether by nerve section or tenotomy, offers protection against degeneration of the fibers, suggests that the vitamin is concerned with the contractile, rather than the resting metabolism of the muscle. The proven fact that the total O_2 consumption of the muscle of vitamin E deficient animals is reduced by alpha-tocopherol, both *in vitro* and *in vivo*, should give a clue to further analysis of its action. Very uncertain still is the importance of vitamin E in the nutrition of human muscle, although it is most unlikely that human muscle will be found to differ fundamentally in its requirements for this vitamin from that of other mammals. As chemical tests are perfected and adapted to clinical use, dietary assays extended, and more is learned about the conditions which govern absorption and utilization, the rôle and importance of vitamin E in human muscle nutrition will become clearer.

For other discussions of the relation of vitamin E to the neuro-muscular systems, the writer wishes to call attention to the recent excellent reviews of H. M. Evans (107), Wolbach and Bessey (108), and Karl Mason (106).

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MICRORESPIRATION TECHNIQUES

JULIAN M. TOBIAS

University of Chicago, Department of Physiology

Microrespirometer techniques have been developed in response to needs for methods of high sensitivity, rapidity of response, or both. The problems approached by these methods may, in general, be grouped into one of two categories:

1. Investigations into the temporal distribution, over relatively long total times, of processes whose progression is mirrored in gas concentration changes. Micromethods are needed for reactions producing a small total change. Stability and sensitivity are equally important.

2. Investigations into the temporal distribution, over short total times, of processes whose progression is mirrored in gas concentration changes. Speed of response and sensitivity are at a premium whereas stability of the measuring instrument becomes progressively less important as observation time shortens.

During the past forty years or so a number of procedures have been developed to deal with such problems as they have arisen in biological researches. It is the purpose of this review briefly to evaluate their relative sensitivities and precisions, and to point out their assets and limitations. The related problems of calibratory techniques and estimation of amount of metabolizing material will also be touched upon.

METHODS OF HISTORICAL INTEREST. *The biometer.* Possibly inspired by the methods of Pettersson and Pettenkofer (1) (2), Tashiro, in 1913–14 (3) (4) (5) (6) (7), constructed an instrument to which he gave the name "biometer". With it he demonstrated that surviving nerve produces carbon dioxide and that the rate of production is increased by stimulation.

A nerve segment was suspended in a CO_2 free, glass respiration chamber. Known amounts of gas could be transferred to an adjoining analysis chamber containing a drop of barium hydroxide whose surface was observed with a lens. If a limiting amount, or more, of carbon dioxide was contained in the sample visible crystals of barium carbonate formed on the drop surface. It had been determined that a "quite definite" amount of carbon dioxide had to be introduced into the test chamber to produce a just visible precipitate. "By determining the minimum volume of any given sample of gas necessary to give the first visible formation of the precipitate, its carbon dioxide content could be estimated accurately, since this volume must have contained just the known detectable amount of carbon dioxide."

Controls indicated that the method was sensitive and quantitatively reliable. For a given construction, the minimum detectable quantity of carbon dioxide was 1×10^{-7} grams. Thus the limiting sensitivity of the method calculates at 0.05 cmm.

Table 1 compares the quantity of carbon dioxide found in 1 cc. of gas with the known composition.

The technique was criticised because data on nerve respiration gave values considerably higher than those found by others. Guttman (8) explained this disagreement in terms of a gush of carbon dioxide following injury to nerve (8) (9) (10) (11) (12). The order of magnitude of the discrepancy, however, seems to invalidate this explanation. It is difficult to reconcile these facts with the results obtained in control calibration.

The biometer, then, was a relatively sensitive instrument detecting 0.05 cmm. of carbon dioxide produced in carbon dioxide free air. It achieved a fairly high degree of precision. When carefully sealed it was stable. Thus it was well suited to the use to which it was put; the detection of a small total change over a relatively long total time.

The Winkler titration. The Winkler titration was adapted to biological experimentation as a method for following changes in concentration of dissolved oxygen. First used by Winkler (13) (14) in a study of oxygen solubility, the method has since been frequently revised and improved. Sources of error have been

TABLE 1

SAMPLE VOLUME REQUIRED TO GIVE PRECIPITATE	WEIGHT OF CO_2/CC. IN GRAMS $\times 10^{-7}$		DIFF. (J. T.)
	Found	Taken	
cc.			*per cent*
1.0	1	0.92	8
0.5	2	2.3	15
0.55	1.82	1.83	0.5
1.5	0.67	0.62	8
2.25	0.45	0.45	0
Average			6.3

pointed out, and modifications of the technique have been devised to deal with them (15) (16) (17) (18) (19) (20) (21) (22) (23). For details one should consult the critical reviews of Theriault and of Theriault and McNamee, and of Allee and Oesting (17) (18) (19).

Lund, having used the method in a study of paramecium respiration (24) adapted it to micro-studies, and (25) was able to handle as little as 5–10 cc. of solution. Thompson and Miller (26) designed apparatus making the micro-titration more convenient and accurate. Using their technique, Snoke (27) compared the micro- and macro-Winkler titrations obtaining the results reproduced in table 2.

In 1933, Kawaguti (28) developed apparatus making possible the titration of as little as 0.1 cc. of solution. Control experiments yielded the results shown in table 3.

Though sources of error were not determined, Kawaguti suggested that corrections could probably be worked out so that the method might be applicable to as little as 0.1 cc. of solution with accuracy.

Allee and Oesting (19) have the following to say about the macro-method: "It

has been our experience that with usual contents of oxygen (N.B. The method is less accurate with very low total quantities of oxygen) we can be sure of repeatability to about 0.03 cc. per litre. Hyman's error, as determined by duplicate determinations, was 0.02 cc. per litre. When the animals being tested consume 2.00 cc. of oxygen per litre the error inherent in the method amounts to from 1.0 to 2.5 per cent. . . . When, however, small differences are recorded the inherent percentage of error mounts." Allee found (29) that a modification, requiring 14 cc. of solution was about as good as the macro-method. Putting these data together one can conclude that for the micro-method, requiring 14 cc. of sample,

TABLE 2

SAMPLE NUMBER	DISSOLVED OXYGEN			
	Macromethod, 250 cc. sample		Micromethod, 4.9 cc. sample	
	Average of 3 determinations	Maximum deviation	Average of 3 determinations	Maximum deviation
	mgm. per liter	*mgm. per liter*	*mgm. per liter*	*mgm. per liter*
1	7.46	0.07	7.42	0.07
2	9.93	0.05	9.91	0.04
3	9.59	0.05	9.64	0.06

(From Snoke—Ecology **10:** 163, 1929.)

TABLE 3

VOLUME OF TEST FLUID	CC./L.	OXYGEN (MEAN OF MORE THAN 11 TESTS) (MAXIMUM DEVIATION)
cc.		
0.5	4.64	0.06
0.4	4.58	0.05
0.3	4.50	0.03
0.2	4.45	0.06
0.1	4.48	0.08
2.0	4.89	0.08
macro-Winkler on 200 cc. sample	5.00	0.01

(From Kawaguti—J. Fac. Sci. Imp. Univ. Tokyo (Zool. sect.) **3:** 183, 1933.)

to be good to 1 to 2.5 per cent the total change in oxygen concentration must be about 28 cmm. or about 2 cmm. per cc. Kawaguti's datum on a 2 cc. sample is also about 2 per cent off the value determined by the macro-method, but on a 0.1 cc. sample the discrepancy is about 10 per cent.

These data indicate that the best one can say for the micro-Winkler titration is that it is capable of detecting a change of 28 cmm. or so of oxygen in a 14 cc. sample of fluid with an error of 1 to 2.5 per cent. Compared to other methods discussed, this is a low sensitivity. In addition, the multiple sources of error and the long time required for an analysis make the method unsatisfactory. It is being, and will doubtless continue to be, largely replaced by the polarographic technique.

The indicator method. In 1916 Haas (30) developed an indicator method for following carbon dioxide production. Since the material producing CO_2 was to be submerged in a solution of an indicator it was essential that the indicator be non-toxic and slowly penetrating. Phenolsulphonephthalein was used. By a comparator method it was possible to measure hydrion concentration changes as small as 1 or 2×10^{-6}. If the determinations were made in pure distilled water, free of carbon dioxide, changes as small as 2 or 3×10^{-8} could be detected. The method was used by Moore (31) (32) (12).

Osterhout (33) modified the technique by circulating the gas, in equilibrium with the solution being studied, through a solution of indicator. Osterhout and Haas (34), and Osterhout (33) (35), and Osterhout's students (Gustafson, Irwin, etc.) modified and used the method in respiration studies.

Lund (36) combined the indicator method with the basic construction of the biometer. Material to be studied was suspended above barium hydroxide containing phenolsulphonephthalein. At given times a known amount of the barium hydroxide solution was titrated with HCl and the CO_2, produced in the intervals, determined.

Parker suspended tissue over barium hydroxide containing the indicator, and frequently renewed the absorbing surface. He was able (9) (37) (38) to confirm the findings of Tashiro on carbon dioxide production by nerve. The sensitivity of the method was estimated to be about one-tenth that of the biometer. The error amounted to about 10 per cent.

The biometer constituted a challenge to those investigators requiring some means of following CO_2 production by submerged tissues. The development of the indicator method answered the challenge with a measure of success. The latter technique was more widely applicable, simpler to use, and could be used for following rates of gas production, which was a more tedious process with the biometer. Osterhout and Haas (22) combined the indicator method with the Winkler titration, making possible simultaneous measurement of carbon dioxide and oxygen in solution.

More recent methods. *The optical lever micromanometer.* The optical lever manometer has, for many years, been known to provide a convenient means of measuring relatively small pressure changes (39) (40). As a macro-instrument it has been chiefly applied in physiological experimentation requiring precise responses to high frequency pressure changes (41) (42).

The incorporation of the optical lever into a micromanometer for biological use was first successfully attempted quite recently (43) (44) (45). In 1939 Heatley, Berenblum and Chain described such an instrument sufficiently sensitive to measure gas volume changes of 1 cmm. per hour.

A paraffin lined chamber of 40 to 80 cmm. ground into a glass plate is so shaped that droplets placed in its several arms may be maintained separate. A steel ball, coated with plastic, is placed in one of the arms. With an electromagnet, this ball can be moved and used as a mixer. The vessel is constructed to allow filling with given gas mixtures. The plate which closes it carries an 18 μ thick mica sheet cemented over a central hole. On either side of the

opening there is another aperture which may be made to coincide with one of the arms of the chamber. A stream of gas can then be circulated through the chamber and the latter subsequently closed by rotating the cover.

The reaction material and reagents are placed in the vessel and the top is closed. A bit of lubricant and a bracket seal and immobilize the mica-glass joint. The upper surface of the mica carries two small plane mirrors which reflect incident beams of light to an observation surface. As the mica sheet becomes depressed or bulges outward, due to absorption or production of gas within the chamber, the reflected beams are displaced. The applied pressure necessary to return the mirrors to their original positions may be manometrically measured, and attendant volume changes calculated.

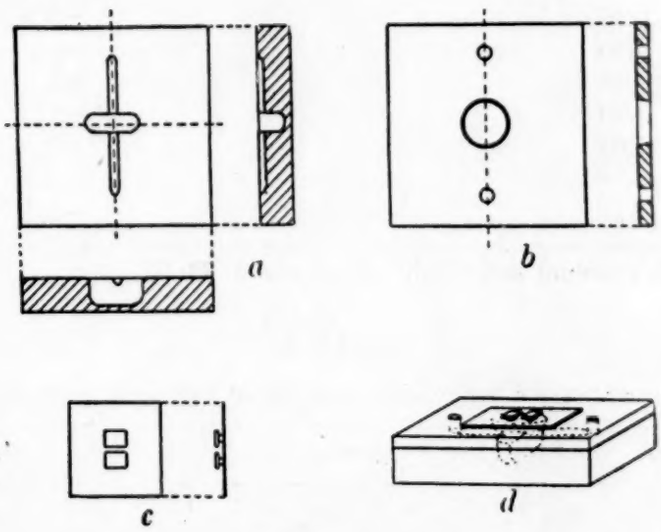

FIG. 1. Components of respiration chamber. *a*, "cup"; *b*, "plate"; *c*, mica membrane with mirrors attached; *d*, complete assembly.
(From Heatley, Berenblum and Chain—Biochem. J. **33**: 53, 1939.)

Accuracy has been determined in several ways. The volume of carbon dioxide displaced from bicarbonate by a known amount of acid has been measured and compared with the calculated value. The results are reproduced in table 4.

Precision was also tested by measuring the amount of oxygen liberated from hydrogen peroxide by catalase. The "known" values were obtained by permanganate titration. The results obtained with the microrespirometer and with the Warburg are reproduced in table 5. In each case approximately 1 cmm. of gas was evolved in the microrespirometer.

The optical lever micromanometer does not possess the sensitivity of most of the other micromethods mentioned. Cumbersome and requiring considerable accessory equipment, it is chiefly notable for the convenient design of the cups allowing it applicability to all the types of problems to which the standard Warburg may be applied. Precision is about equivalent to that of the Warburg technique. A number (up to six for the present design) of the respirometers can be used at one time (45). Thermobarometric controls afford relatively high stability for long time measurements.

TABLE 4

VOLUME CO_2 EVOLVED (CALC. VALUE = 0.90 CMM.)	ERROR
cmm.	*per cent*
0.83	−8
0.85	−5.5
0.90	0
0.82	−9
0.87	−3.5
0.84	−6.5
0.87	−3.5
0.85	−5.5
0.90	0
0.96	+6.5
0.87	−3.5
0.89	−1
0.88	−2
0.89	−1
0.92	+2
Average.....	−2.7

(From Heatley, Berenblum and Chain—Biochem. J. **33:** 53, 1939.)

TABLE 5

Volume oxygen per cubic centimeter of hydrogen peroxide

MICRORESPIROMETER	WARBURG	CALCULATED
204		202
208		
198		216
200		
78		
77	78	
77	81	
77	81	
80		
81	77	
74	77	84.5
75	78	
79	79	
79	77	
85	80	82.5
79	81	
82		

(From Heatley, Berenblum and Chain—Biochem. J. **33:** 53, 1939.)

The Cartesian diver. A considerable volume change occurs isothermally in systems in which certain chemical reactions take place (46). Taking advantage of this fact, Linderström-Lang (47) (48) and Linderström-Lang and Lanz (49) (50) adapted the falling drop method for determining specific gravity to studies in enzymatic histochemistry. The technique was modified from that used by Barbour and Hamilton (51) in the estimation of specific gravity, and by Vogt and Hamilton (52) and by Fenger-Eriksen, Krogh and Ussung (53) in the estimation of deuterium oxide.

A floating object comes to rest in a fluid column of graded specific gravity at such a point that the specific gravities of the two are equal. This fact underlies both the gradient tube technique and the cartesian diver method. Though the gradient tube has been used only for the study of volume changes in liquid systems the cartesian diver has been used in microgasometry. A few words about the gradient tube seem appropriate here.

In a vertical glass tube, at a constant temperature, a practically linear specific gravity gradient can be produced by properly mixing kerosene and bromobenzene. A droplet of a watery solution placed beneath the surface of the mixture, which has been previously saturated with water at a suitable vapor pressure, will fall and find an equilibrium position where the specific gravity of medium and drop are equal. If, however, reactions occur within the drop, producing a volume change, the drop will not come to a standstill but will continue to fall or begin to rise at a rate which, under certain conditions, is a measure of those processes.

The gradient in the kerosene-bromobenzene column is measured by noting the positions at which test drops of known density come to rest. Drop movement is followed with an ocular micrometer, and specific gravity is ascertained by comparison with the positions of standard drops. The drops being small and on all sides surrounded by fluid come almost immediately to temperature equilibrium.

The accuracy of the density measurements is 1 to 3 in the sixth decimal place. Since 0.1 cmm. drops can be used, this corresponds to a volume change of about 1 to 3 $\times$ 10^{-7} cmm. which, it is claimed, can be measured within a few per cent of error. Such a combination of extreme sensitivity and high precision has been demonstrated for none of the other techniques mentioned in this review.

First described in terms of its usefulness in biological experimentation by Linderström-Lang (54), the cartesian diver technique has been extended by Linderström-Lang and co-workers and by Boell and co-workers (55) (56) (57) (58) (59) (60) (61) (62) (63).

A glass vessel (diver) of some 10 to 30 (occasionally up to several hundred) cmm. is stoppered with an oil droplet free to move in the neck of the vessel. Submerged in a fluid medium the vessel quickly comes to equilibrium position. Should the inside pressure change, the oil droplet and outside fluid respond by moving in the neck, and depending on whether total volume increases or decreases the vessel rises or falls in its external medium. The pressure which

must be applied to return the diver to its original position can be measured and translated into volume change.

In his original paper Linderström-Lang stated that "any variation of the quantity of gas in the diver may be measured with an accuracy of $\frac{10 \times 0.02}{100}$ cmm." (54). Boell, Needham and Rogers set the sensitivity at 0.001 cmm.

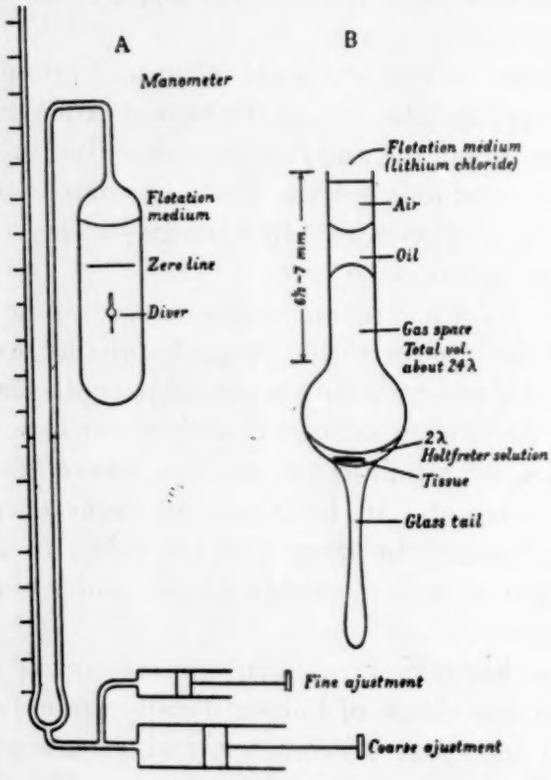

FIG. 2

(From Boell, Needham and Rogers—Proc. Roy. Soc. London B 127: 322, 1939.)

The equilibrium pressure is said to be reproducible within 1 to 2 mm. of water (the diver can be made to stay at the mark for ten seconds or so). If v be the gas volume of the diver and p the equilibrium pressure, then

$$\frac{\Delta v}{v} = \frac{\Delta p}{p}$$

Since p and v are of the order of magnitude of 10,000 mm. and 10 cmm. respectively, an accuracy of pressure reading of 1 to 2 mm. of water corresponds to an accuracy of volume measurement equal to 0.001 to 0.002 cmm. From this one would expect that, were the total volume change only 0.001 to 0.002 cmm., the error to be expected to its immediate measurement would be about 50 to 100 per cent.

Boell, Needham and Rogers studied cystein oxidation and compared diver measurements with Warburg results. They state that "in the case of cystein oxidation rate the average diver results were 106 per cent of the Warburg results; in the case of cystein oxidation end values they were 99 per cent, and in the case of yeast fermentation 102 per cent".

These same workers have published a graph relating oxygen uptake by cystein oxidation to time. Though only approximately, oxygen uptake can be tabulated against time as read from the points on this graph. If such an approach is not too crude one may conclude that, for gas volume changes of an average of 0.0026 cmm. *each* reading of such a volume change may, on the average, be 70 per cent inaccurate. This conclusion would be in approximate agreement with the estimate of Linderström-Lang already cited. On the other hand, when the volume change between readings is of the order of 0.04 cmm. the deviation is considerably less.

The technique is thus seen to be quite sensitive (to about 0.002 cmm.). The inclusion of thermobarometer divers allows an experiment to be continued over many hours with a high degree of stability. For minute to minute measurements of volume changes of the order of 0.001 cmm. precision is poor. Interpolation of such volume changes from larger total changes is very accurate. It is probable that with a smaller diver than that which is commonly used, and with the exercise of extreme care in setting the diver at the reference mark greater accuracy in the immediate estimation of very small volume changes could be attained.

The polarograph. The studies of Kucera, in 1903 (64), on the electrocapillary curve of mercury led to the development of the polarograph by Heyrovsky (65) and by Heyrovsky and Shikata (66).

The method depends on the fact that solutes (electrolytes or non-electrolytes) are reduced (or oxidized) when a current is passed through their solutions at voltages above their decomposition potentials. The current flowing at such a potential (half-wave potential) depends on the rate of diffusion of the solute to the electrode, and this rate depends, in turn, upon its concentration. Current measurements give quantitative data on solute concentration.

In the usual polarographic determination the dropping mercury electrode is used. For details of the method and for the theory underlying it one should consult the work of Kolthoff and Lingane (67) (68).

The technique has been applied to the estimation of oxygen in solution and is far superior to the Winkler titration. The possibility of thus using it dates back to work of Vitek in 1933 (69). It has since been used in a number of biological studies (70) (71) (72) (73) (74).

Objection to using the method with living tissue has stemmed chiefly from the fear that metallic mercury in contact with the medium might poison the material being studied. In 1940, Kolthoff and Laitinen (75) showed that oxygen in solution can be polarographically determined using a platinum wire microelectrode in place of the dropping mercury electrode. Papers of Laitinen and Kolthoff (76) (77) published in 1941 should be consulted for detail.

Davies and Brink (78) described a microrespirometer incorporating the platinum microelectrode. A bright platinum or platinum-iridium wire (about 27 μ in diameter) is placed in circuit with a calomel half-cell by way of a glass tube containing tissue and its medium. The platinum electrode is made 0.9 volt negative with respect to the half-cell, and the current which then flows is

proportional to the oxygen concentration of the fluid. A sensitivity to 0.001 cmm. volume change of oxygen was attained.

The stationary platinum electrode does not come immediately to equilibrium with the solution in which it is placed. Current intensity gradually changes for from two to ten minutes (75) (76) (77) (78). In certain instances this defect has been overcome by rotating the electrode (75) (77).

The prospect of even greater sensitivity, great speed of response if the "seeking" electrode is close enough to the source of changing oxygen concentration, relative indifference to temperature changes, high precision, and convenience of operation make this instrument one which will probably have very wide applicability in microrespirometry. The electrode has also been directly inserted into the tissue being studied (78a).

The capillary microrespirometer. The capillary microrespirometers to be discussed are all built, more or less, about the same basic design.[1] Essentially there are two chambers, one of which contains the biological material, its medium and specific reagents for the absorption of certain gases. The other contains medium and reagents alone. The two chambers are connected by capillary tubing carrying a fluid index droplet. Gas volume change in the tissue chamber creates a pressure difference along the capillary lumen, in response to which the index drop moves. Droplet movement is recorded and timed, and these data are translated into volume change.

First used by Thunberg in 1905 (79) (80) (81), the capillary micromanometer was a gas analysis apparatus of the Pettersson type (1) permitting introduction of the animal or tissue into the measuring pipette. Change in volume of enclosed air indicated the difference between oxygen absorbed and carbon dioxide produced. The air was then passed over potassium hydroxide for carbon dioxide absorption. Later, Thunberg (82) (83) modified the apparatus using equal volume respiration and compensation chambers, each about 3 cc., with a capillary of 0.2 to 0.1 mm. diameter. This was the first instance of the use of a compensation chamber in a respiration apparatus. The movement of a petroleum index droplet was observed by the naked eye against a millimeter scale. An upward convex capillary allowed the index to come always to the same equilibrium position. Modified by Winterstein (84), the apparatus was used in further respiration studies. The ratio of compensation to respiration chamber volume was unity. A volume change of about 1.4 cmm. could be measured. Nothing was recorded concerning regularity of movement of the index. During the next few years the apparatus was used in a number of laboratories (85) (86) (87). In 1912–14 Winterstein (88) (89) (90) again modified the apparatus by incorporating a mercury manometer.

The index being set at zero the level of the mercury column was noted. When

[1] The Barcroft and Warburg manometric techniques and the variations thereof are not being considered in this discussion, because they have been so carefully and exhaustively treated by Dixon in his book on *Manometric Methods* (Cambridge University Press, 1934). A further development was described by Summerson in the Journal of Biological Chemistry vol. 131 (1939), p. 579.

a reading was to be taken the droplet was brought to zero position by means of a screw clamp. This simplified things considerably. It was not necessary to know chamber volumes or capillary diameter to translate to gas volume change. A somewhat later model than the one described, allowing the chambers to be filled with any given gas mixture, was used by Wolf (91).

Krogh (92) (93) (94) (95) modified the Barcroft blood gas apparatus, in 1914, to serve as a micromanometer. Chambers communicated by way of a 0.4 to 0.5 mm. internal diameter U-tube. The index was kerosene, and the manometer itself was outside the thermostat. Only the "reinste Petroleum" was used, but nothing was said about regularity of meniscus movement for small pressure changes. Krogh stated that the Winterstein apparatus could "be made still more sensitive but (it) is not as well suited to prolonged experiments" as his own. This comment stems from the fact that the Winterstein apparatus incorporated a very short capillary, hardly a serious difficulty.

In 1920, Adam (96) combined Winterstein's mercury burette with the Krogh manometer. Paraffin oil of B.P. 160° to 190°C. served as index. Volume changes of 20 cmm. per hour could be measured with 2 to 3 per cent accuracy. Bass (97) introduced the horizontal microscope for following the index. Bodine and Orr (98) employed a slightly modified Krogh manometer.

In 1925 Novy, Roehm and Soule (99) made the next improvement in this type of microrespirometry. Up to that time the ratio between compensation and respiration chamber volumes had always been close to unity. Novy, however, pointed out that "Sensitivity of the closed end manometer varies directly with the volume of air in the sealed end and inversely as the square of the radius of the manometric tube". A gain in micromanometric technique, this modification was not taken advantage of until some years later when Gerard (99a) utilized the same principle in the construction of a modified Warburg apparatus.

Fenn (10) devised a capillary micromanometer which he himself then found to be (100) "essentially similar to Thunberg's original design". Though the principle of large compensation chamber opposed to smaller respiration chamber was not incorporated, the instrument has been used by many investigators. The smallest capillary was about 0.3 mm. i.d. Meniscus position was read to 0.1 mm. with a hand lens so that the smallest appreciable volume change was about 0.03 cmm. In 1935 Fenn wrote a detailed paper on the theory and technique of the method (100).

The differential volumeter was now used by a number of workers (101) (102) (103) (104) (105) (106) (107) (108), but none of these described any attempt to improve sensitivity. Very small movements of the index were not measured, and neither irregularities in the movement of the droplet nor deformity of the meniscus became important. Consequently no detailed study of index fluid was made.

In 1933 Schmitt (109) further developed the capillary microrespirometer to a point where it was sensitive to a 0.0005 cmm. volume change. A large compensation chamber was not used though both vessels were of relatively large absolute volume. Projected experiments made it desirable to make observa-

tions on index movements over very short periods of time. To locate the droplet a traveling horizontal microscope was used. Considerable attention was paid to the selection of index fluid. After trials with dimethyl aniline, butyric acid, valeric acid, and others, highly purified kerosene was finally used for the majority of experiments. Even so there were occasional difficulties due to residual resins.

It was next found that to get a smoothly moving droplet, over periods of time as short as three minutes, it became necessary to go to extremes in temperature control. Finally, with a thermostat accurate to "better than 0.001°C." a smoothly moving meniscus was observed. For 10 mgm. of nerve (wet weight) readings were consistent to 2 to 3 per cent even if made as frequently as every two minutes. Such precise thermoregulation was probably necessary because of the relatively large chambers used and by their rather wide separation in the bath.

Schmitt pointed out that basal drift of the index might be due to slipping at ground joints, or to unequal evaporation of the droplet at its two surfaces. Jamming the stoppers and wetting the capillary walls before closing the bridge cock, made it possible to diminish equilibration time to half an hour or less. Capillary diameter was 0.4 to 0.5 mm.

Thus with a 16 mm. objective and a 6× ocular, the apparatus was sensitive to a volume change of 0.0005 cmm. Precision to 2 to 3 per cent with a total volume change of about 0.02 cmm. was attained. This volumeter has also been used by Hill (110).

A capillary microrespirometer of different design has been described by Stefanelli (111) (112). The chief innovations were a reduction of chamber size to 80 cmm., and the incorporation of a glass window to allow observation of tissue. The instrument was sensitive to a volume change of 0.003 cmm.

Howland and Bernstein, in 1931 (113), studied the respiration of a single paramecium by the capillary method. It is interesting that with their comparatively crude apparatus they found the oxygen consumption of a single Paramecium caudatum to be about 0.0005 cmm. per hour, and it was only in 1940 that Boell and Woodruff (62) with the cartesian diver technique, determined the value for the Paramecium calkinsi to be 0.00048 cmm. per hour per organism.

The chief importance of the work of Bernstein and Howland is derived from the finding that if one heats capillaries sufficiently to seal them they require many hours to cool. This is, of course, also dependent upon the efficiency of the thermostat employed and the thickness of the capillary walls. The point is pertinent concerning the earlier work of Kalmus who (114) (115) made respirometers by drawing tubing out to capillary dimensions at one end. The material to be studied was sucked in and followed by a drop of paraffin oil. The end was heat sealed. The fine capillary tip was next thrust through the surface of a 10 per cent KOH solution and then into paraffin oil. The interface between the alkali and oil was followed with an ocular micrometer. This work must have been highly unprecise due to the effect of passing the measuring

capillary through a fluid surface with its adsorbed contaminants. The same objection can be raised to the work of Pearce and Gerard (116).

In 1934 Gerard and Hartline used both a very small respiration chamber (about 60 cmm. volume) and a very large compensating chamber (117). The small respiration chamber greatly increases stability. Drop movement irregularities are due, among other things, to temperature fluctuations. Thermal movement of the droplet is minimized as volume is decreased, since in this case volume change is proportional to total volume. To prevent drop movements due to changes in atmospheric pressure the manometer is closed off from the air. Instead of achieving this by balancing the respiration with an equal volume compensation chamber, these workers enclosed the entire measuring capillary in a jacket of relatively large volume. Thus sensitivity was not sacrificed for stability, and the achievement of the latter was, at the same time, simplified. The ratio of control volume to respiration chamber volume being about 1000:1 the

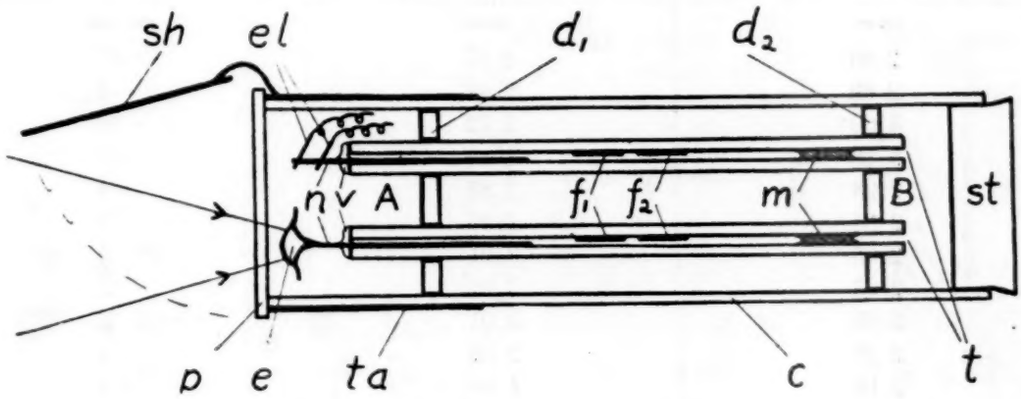

FIG. 3. f_1 and f_2—filter papers with acid and alkali; *m*—index droplets; *v*—vaseline seals; *n*—tissue; *st*—rubber stopper.

(From Gerard and Hartline—J. Cell. and Comp. Physiol. **4**: 141, 1934.)

volume change represented by any given droplet movement was effectively the completely undamped volume change (the correction factor being 1/1000).

Temperature control was found to be simplified much beyond the elaborate apparatus employed by Schmitt. Capillaries of 0.5 to 1.1 mm. internal diameter were used with a droplet of isotonic NaOH as index. Index movement was followed by means of a horizontal microscope. Volume sensitivity achieved was about 0.0013 cmm. For minute to minute figures to be reproducible, the total volume change had to be about 0.02 cmm. Reproducibility was therefore just as good with this simple technique as with the apparatus of Schmitt.

Since this work the basic design of the instrument has been used by a number of workers some of whom have modified and adapted it to special problems (118) (119) (120) (121) (122) (123) (124) (8) (116).

Recently several attempts have been made to incorporate some sort of device with the capillary micromanometer so that reagents could be mixed during an experiment.

Thimann and Commoner (125), in 1940, made a contribution toward this end. Using small capillaries (0.2 to 0.3 mm. i.d.) they arranged the apparatus

to allow for the addition of substrate during an experiment. Measuring droplet movement to 0.1 mm. these authors attained about the same sensitivity as previous workers. However, reading the meniscus to the nearest 0.1 mm. means an average error of 10 per cent per mm. of movement or 0.006 cmm. for a volume change of 0.060 cmm. Thus for volume changes of 0.006 cmm. (which is of the order of magnitude of sensitivity of most of the constructions discussed) the error would be about 50 per cent, on the average, and for volume changes of 0.001 cmm. it would be about 400 per cent. This sort of error could easily be obviated by more accurately determining meniscus position.

Cunningham and Kirk have made an effort in the same direction (126). Side by side, in a brass block, there are placed respiration chambers which, by way

TABLE 6

VOLUME CO_2 LIBERATED (CALCULATED)	VOLUME CO_2 LIBERATED (MEASURED)	ERROR
cmm.	*cmm.*	*per cent*
2.60	2.37	−7
2.60	2.48	−5
3.00	2.72	−10
3.15	3.12	−3
2.98	2.85	−4
2.38	2.46	3
2.95	3.01	2
2.95	3.01	2
2.95	3.01	2
2.16	2.09	−3
2.16	1.99	−8
2.16	2.08	−4
3.42	3.60	5
2.16	2.08	−4

Average per cent error = ±4.5
Average per cent recovery = 98.0

(From Cunningham and Kirk—J. Gen. Physiol. **24:** 135, 1940.)

of hollow vertical pillars, communicate with each other through a glass capillary. Kerosene serves as index fluid. The authors state that "The symmetrical construction and the placing of the chambers inside a metal block insure a uniform temperature distribution, permit the elimination of the thermostat, and make possible a very high degree of sensitivity." Droplets are placed on the floor and roof of the chamber and are mixed by drawing a bit of glass covered metal from one to the other with an electromagnet.

Liberating known amounts of carbon dioxide within the chamber, the following calibration data were obtained.

Reproducibility was also tested by measuring paramecium oxygen consumption.

Table 6 shows the error to be 4.5 per cent when the total volume change is, on the average, 2.7 cmm. In table 7 the average of the probable errors,

referred to millimeter displacement per minute, is 8 per cent of the average displacement. This is large considering that average displacement per minute is 0.3 mm., and readings are made at 10 to 20 minute intervals. The chief reason for this lack of precision probably stems from the fact that the capillary is exposed to air currents and the gas may well be at different temperatures on the two sides of the index. Even in a constant temperature bath Schmitt found that temperature pockets could cause serious trouble (109).

TABLE 7

EXPERIMENT NUMBER	TRIAL NUMBER	OXYGEN CONSUMPTION		
		mm. displacement per minute		cmm. O_2 at N.T.P./hr.
1	1	0.30*		
	2	0.26	0.26 ± 0.04	0.66 ± 0.10
	3	0.25		
	4	0.23		
2	1	0.32*	0.33 ± 0.02	0.85 ± 0.05
	2	0.35		
3	1	0.20†		
	2	0.22	0.21 ± 0.01	0.54 ± 0.02
	3	0.22		
4	1	0.30*	0.29 ± 0.01	0.74 ± 0.02
	2	0.28		
5	1	0.14*	0.14 ± 0.01	0.35 ± 0.02
	2	0.15		
6	1	0.10†	0.09 ± 0.01	0.23 ± 0.02
	2	0.08		
7	1	0.53*		
	2	0.51	0.53 ± 0.05	1.35 ± 0.13
	3	0.58		
	4	0.50		

* Measurements made at 10 minute intervals.
† Measurements made at 20 minute intervals.
(From Cunningham and Kirk—J. Gen. Physiol. **24:** 135, 1940.)

On the other hand the apparatus has these advantages: the sensitivity can be readily altered by substituting capillaries of varying sizes; chamber volume can be readily altered; advantageously placed outlets allow for filling the chambers with various gas mixtures; two solutions may be mixed during an experiment. In a more recent paper Barth and Kirk have described a modification of this method (126a) in the direction of considerable improvement.[2]

In 1941 Tyler and Berg (127) described a somewhat different capillary microrespirometer. A mercury piston was introduced to permit returning the index

[2] Footnote appears on page 75 at end of References.

drop to its zero position for each reading. This device allows estimation of volume change from measurement of the amount of mercury introduced. It will be recalled that Winterstein, in 1912 (88) (89), and Duryee, in 1936 (119), described similar instruments.

It is stated by Tyler and Berg that their readings are not affected by overall temperature changes, but that it is essential to avoid temperature difference on the two sides of the drop. Since this latter is far more difficult to achieve than is a constant overall temperature the net advantage is slight (109). The instrument has the advantages that tissue being studied can be seen during an experiment (see also Stefanelli (111)) and substrate or other reagent can be added to the metabolizing material. The authors predict an error of 10 per cent for a volume change of 0.003 cmm., but state that this precision has not been attained. Experience indicates that such accuracy can be achieved with care in the selection of index drop fluid (109) (128).

Following the use of the katharometer by Shakespear (129) and by Shakespear and Daynes (130), Daynes (131) detailed the mathematical theory of the instrument. Slater (132) combined the katharometer with the capillary microrespirometer. Oxygen consumption was measured volumetrically and carbon dioxide production katharometrically. The instrument could be used to measure an oxygen volume change of 1 cmm. and a carbon dioxide volume change of 0.5 cmm. The technique has not been applied further in biology except in the macrometabolism studies of Ledig and Lyman (133) and Rabinowitch and Bozin (134).

Fenn (10) combined a conductivity cell with the differential volumeter. Described in detail by Fenn (10) (100) and briefly discussed by von Ledebur (135) this method has received but little attention.

Tobias and Gerard (128) further developed the capillary respirometer so that it is now convenient to follow the respiration of as many as ten or more tissue samples at once. This number can easily be expanded if necessary. Gas volume changes of the order of 0.0005 to 0.001 cmm. per minute can be followed at minute to minute intervals with individual readings varying from the mean by an average of 8 per cent. Over total times of five to ten minutes the interpolated minute values have a higher precision, probably about 2 per cent.

Each respirometer consists of a 90 mm. length of 0.2 mm. capillary tubing sealed, with de Khotinsky cement, into a 30 to 40 mm. length of 1.2 to 1.5 mm. thin walled tubing. The latter segment serves as tissue and reagent chamber. The smaller capillary carries the index drop. After inserting tissue and absorbing reagents the open end of the chamber is sealed with plasticine. A number of units is mounted around a central heavy walled capillary extending from a glass stopper. The stopper is placed in its sheath, and the whole, submerged in a water bath, is supported in such a fashion that it can be rotated about its central axis. Thus, successive capillaries and their indices can be brought into the field of a horizontal microscope.

The utilization of a very small respiration chamber (30 to 50 cmm.) opposed to a large outer chamber and enclosed therein affords great stability. The chief

advantages of the technique are this stability, high precision for very small volume changes, and the convenient multiple tube arrangement. The chief disadvantages stem from the fact that the capillaries are very fragile, and thus must be measured for each experiment and discarded. This is far too time consuming. Up to this point also it had not been possible to add substrate to the tissue during an experiment nor to observe the tissue.

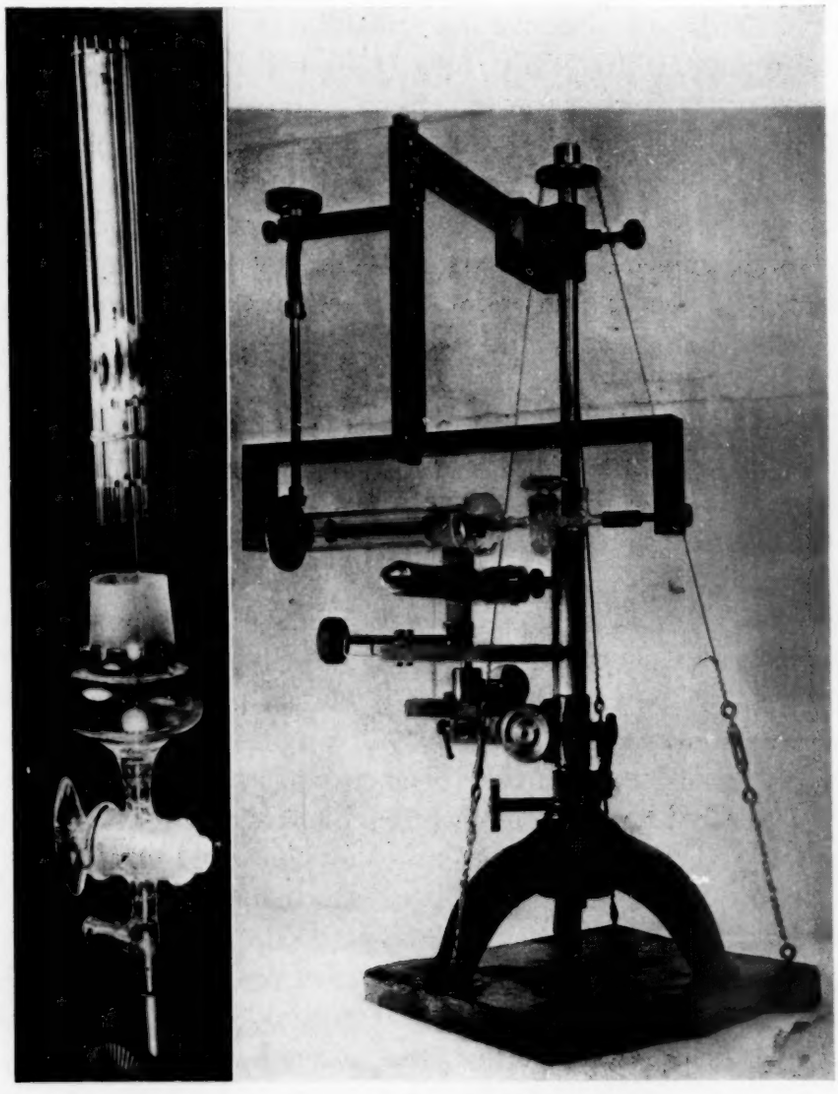

Fig. 4

In order to overcome these disadvantages the instrument has been modified. Permanent, heavy-walled capillary units, expanded at one end to accommodate tissue and absorbing reagents, replace the fine capillaries. A small ground glass cap instead of plasticine closes the chamber. The units are mounted in a vertical bank inside a brass-glass box. Brass clips into which the respirometers fit are rotatable from the outside of the box by means of a metal arm connected thereto by a packed joint. Since the tissue being studied clings to the roof of the expanded end of the unit, a droplet of substrate to be added may be placed on the floor along with a small lead shot coated with celloidin and paraffin.

Rotation of a single capillary at any time then causes the mixing ball to drag the reagent around to the tissue. Greasing of the cap-respirometer joint makes it transparent and thus allows visual observation of the tissue being studied.

In 1942 Scholander described (138a, 138b) a volumetric respirometer sensitive to about 0.01 cmm. per hour. It embodies principles similar to those utilized by Duryee and by Tyler and Berg.

Miscellaneous techniques. From time to time micromethods have been developed for gas analyses based on the principles of selective absorption of gases by specific reagents (136) (137) (138) (139) (140) (141) (142). Perhaps the most noteworthy applications to biological problems were those of Krogh who, following the lead of Timiriazeff (143), developed the tonometric method (94) (144) (145) to a high degree of sensitivity and precision (146) (147).

The technique used by Hill (148) in a study of rubber permeability has not been otherwise applied. It involved the detection of oxygen by luminous bacteria which can be seen to glow when oxygen partial pressure is as low as 0.0053 mm. Hg. Other such data on minimal required oxygen concentration have been obtained (149) (150).

Dublin, Boothby, Brown and Williams (151) analyzed mixtures of He, O_2 and N_2 by determining sound velocity in the mixture. The method has no qualitative value. No effort has been made to adapt it to micro-studies, and it is unlikely that such adaptation will ever be made because of limited sensitivity.

McAlister (152) developed a spectrophotometric technique for the study of plant respiration and photosynthesis (153) (154). In 1939 Pfund (155) modified the method by the introduction of a phonic interrupter wheel and pitch detector. Simple calculations show that an adaptation of the method to micro levels, of the order discussed in this paper, would involve very serious technical difficulties. No effort has been made at such an adaptation.

Interferometry has been applied to problems in metabolism (156) (157) (158). The great cost of the apparatus and its relative complexity have overshadowed the assets of great precision, speed and sensitivity.

Utilizing the interferometer principle (159) a sensitive indicator of pressure change has recently been developed by the author. To the surface of a microscope slide there is fastened, with shellac, a cover slip (about 0.1 mm. thick) through which has been drilled a hole (convenient size for preliminary experiments has been about 2 mm. diam.). Over the hole a thin organic film is spread and is held in place by another, similarly drilled, cover slip and shellac. Multiple reflections of monochromatic light in the gap between the cover slip and the microscope slide produce circular, concentric interference fringes. As pressure change causes the membrane to be displaced with respect to the fixed slide the fringes are seen to shift. Because of the extreme thinness of the film the instrument is very sensitive. (Neither the film nor the slide need be silvered or aluminized. Reflectivity is high enough to produce bright, clear fringes.) The instrument responds to pressure changes of the order of 0.05 mm. water (about 0.004 mm. Hg).

Summary of sensitivities. Recently several authors (57) (160) have published lists of so-called sensitivities for the various microgasometric methods. These have expressed sensitivities in terms of cubic millimeter volume change measured by a given shift of meniscus position, in the case of the manometric methods. Such lists assign different sensitivities to different constructions of capillary respirometers incorporating capillaries of different dimensions and utilizing different methods for reading meniscus position. Obviously these distinctions are arbitrary.

Table 8 gives the limits to which the sensitivities of the various genera of apparatus have so far been pushed. Several of them are capable of development to considerably greater sensitivity. Errors estimated are those which might reasonably be expected were the indicated volume change measured only once.

TABLE 8

INSTRUMENT	MEASURES	SMALLEST MEASURABLE VOLUME CHANGE—ESTIMATED DIRECTLY FROM TWO READINGS	INTERPOLATED FROM SERIES OF MEASUREMENTS	ERROR OF METHOD AS DESCRIBED IN TEXT
Biometer	CO_2	2.5×10^{-2} cmm.		6%
Winkler titration	Dissolved O_2	28 cmm.		1–2.5%
Indicator	Dissolved CO_2	5×10^{-1} cmm.		10%
Optical lever	CO_2, O_2, etc.	1 cmm./hour		2–3%
Gradient tube	Volume change in liquid systems	3×10^{-7} cmm.		A few %
Cartesian diver	CO_2, O_2, etc.	$1–2 \times 10^{-3}$ cmm.		50–100%
		5×10^{-2} cmm.		A few %
			$1–2 \times 10^{-3}$ cmm.	A few %
Polarograph	Dissolved O_2	1×10^{-3} cmm.		Very precise
Microcapillary	CO_2, O_2, etc.	5×10^{-4} cmm.		8%
			5×10^{-4} cmm.	A few %

Reactions suitable for the calibration of various microrespirometers. Numerous reactions, proceeding at a known rate, have been utilized for the calibration of micromanometric systems of various sorts. Some of these, such as the liberation of carbon dioxide from bicarbonate by excess of acid, and the fermentative processes need no additional mention. Some other suitable reactions are noted below with references. Not all have been used for the purpose indicated.

Oxidation of cysteine

Harrison. Biochem. J. **18:** 1009, 1924.
Boell, Needham and Rogers. Proc. Roy. Soc. London (B) **127:** 322, 1939.
Kharasch, Legault, Wilder and Gerard. J. Biol. Chem. **113:** 537, 1936.

Decomposition of oxaloacetic acid

Ostern. Hoppe Seyler's Ztschr. **218:** 160, 1933.
Boell, Needham and Rogers, as above.

Decomposition of acetoacetic acid

Ostern, as above
Boell, Needham and Rogers, as above
Ljunggren. Ber. d. deutsch. Chem. Gesell. **56:** 2469, 1923.
Ljunggren. Biochem. Ztschr. **145:** 422, 1924.
von Euler u. Olander. Ztschr. anorg. Chem. **147:** 295, 1925.
Burki und Schaaf. Helv. Chim. Acta **4:** 418, 1921.
Widmark. Acta med. Scand. **53:** 393, 1920.

Decomposition of alpha-alpha-dimethylacetoacetic acid

Pedersen. J. Am. Chem. Soc. **51:** 2098, 1929.

Decomposition of nitramide

Bronsted und Pedersen. Ztschr. physik. Chem. **108:** 185, 1924.

Decomposition of nitroso-triacetone-amine

Bronsted and King. J. Am. Chem. Soc. **47:** 2523, 1925.
Francis and Gliddens. J. Chem. Soc. **101:** 2358, 1912.
Francis and Geake. J. Chem. Soc. **103:** 1722, 1913.
Francis, Geake and Roche. J. Chem. Soc. **107:** 1651, 1915.

Peroxide decomposition by catalase

Heatley, Berenblum and Chain. Biochem. J. **33:** 53, 1939.

Cathodal reduction of oxygen

Davies and Brink. Proc. Am. Physiol. Soc. 69, 1941.

Estimation of amount of metabolizing material. Since, for purposes of comparison, it is necessary to refer respiration intensity to the amount of respiring material present, the accurate estimation of the latter becomes an important factor in determining the significance of such data. The problem is equally difficult in macro and micro studies from the point of view of partitioning respiration intensity amongst the various cellular constituents present in a bit of tissue. As tissue homogeneity increases, this matter becomes progressively less troublesome. In micro-studies there is the more primitive problem of simply determining the total amount of material present.

The commonest method for getting at the amount of respiring material involves the determination of dry weight. The ordinary Kuhlman microbalance can be used for dry weights of 80 μg or more (44). With smaller amounts of tissue serious error can be introduced. For weights of less than 80 μg the Donau microbalance (44) (161) is recommended. Torsion balances sensitive to 0.002 mgm. are commercially available. For weights of the order of 0.0001 mgm. the quartz fiber balance should be used. In 1941 Lowry described such a balance with a sensitivity of about 0.03 γ, and with reproducibility of 0.1 γ. The maximum load capacity was about 250 γ. A modified type of quartz fiber balance has also been described by Bazzoni (169) (170).

Total nitrogen determination can be done accurately on small amounts of tissue, and nitrogen content can be used as the standard of reference. Like the

measurement of dry weight, this method does not differentiate between respiring and non-respiring material. Referring respiration intensity to dry weight may also give a false picture of the actual situation since various organs and tissues and parts of organs have quite different water contents.

Nucleic acid determination may be used to differentiate cellular from non-cellular material (44). After suitable extractions nucleic acid phosphorus may be determined by the method of Berenblum and Chain (162).

Recently Caspersson (163) has developed an ultraviolet absorption method for the determination of nucleic acid compounds which have a characteristic absorption curve in the ultraviolet spectrum. The curve can be gotten on microscopic sections or smears. Its shape identifies the compound, and the height of the curve can be used as a measure of the amount present in the field measured. This method has not, to date, been used in conjunction with respiration studies, though it has been used to follow the intracellular metabolism of nucleic acid (164).

Cell counts are, of course, ideally suited to the study of cell suspensions. Recently the use of counting techniques has received a certain impetus in the study of solid masses of tissue also. Glick, Holter, Linderström-Lang and Seberg Ohlsen (165) developed a cell counting technique in connection with studies on enzymatic histochemistry. The method consists of cutting frozen sections from a core of tissue using alternate sections for histological and enzyme analysis. The authors point out that counting nuclei is much preferable to counting cell outlines. It may be noted that when nucleoli are present as single and constant structures they would afford a still better index of number of cells (166) (167).

For muscle, Grossman (168) has found that maceration in 5 per cent citric acid causes extrusion of nuclei. These might then be taken up, homogenized by shaking and counted in a hemocytometer chamber. Recently Pearce and Gerard (116) have macerated tissue by grinding with a small glass pestle in concentrated acetic acid. After maceration the tissue is stained, shaken in a blood pipette, and nuclei are counted in a hemocytometer chamber.

The measurement of tissue volume has also been attempted (128). Tissue is forced into a capillary of known diameter and the length of the cylinder it occupies is then measured directly. Adequate data for evaluation are not yet available.

The improvement in microgasometric techniques has gone far enough so that use of bits of tissue sufficiently small to allow direct counting of all cells present is feasible (166). Thus, in contrast to the work of Glick et al., cell counts can be made directly on the sample which has been studied metabolically. This would also allow for measurements of cell and nuclear dimensions. Such work is, at present, in progress in this laboratory.

In conclusion, it is important to emphasize that none of these methods affords a basis for the apportionment of total respiration intensity amongst the various cell types present in a given piece of tissue. The importance of knowing something of this distribution is great, and the problem presents a very real challenge to workers in the field.

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[2] Since this review has gone to press Cunningham and Kirk (Jour. Cell. and Comp. Physiol. **20:** 119, 1942) have published a paper describing a capillary respirometer sensitive to 5×10^{-5} cmm. The respirometer is of the open type and represents a development of the Kalmus technique. The criticisms levelled at the Kalmus method have been circumvented.

INTERRELATIONS OF CALCIUM AND ASCORBIC ACID TO CELL SURFACES AND INTERCELLULAR SUBSTANCES AND TO PHYSIOLOGICAL ACTION

MARY ELIZABETH REID

Division of Chemotherapy, National Institute of Health, U. S. Public Health Service, Bethesda, Md.

A better understanding of the physiological effects of vitamins in plants and in animals is a goal toward which a number of different lines of investigation are now being directed. Some insight has been gained concerning the mode of action of several of these substances in animals but comparatively little is known of their functions in plants. It has been demonstrated that several members of the vitamin B complex participate in respiratory processes and because of its reducing action, it has been suggested that ascorbic acid also is concerned in respiration. There is as yet, however, no considerable amount of evidence clearly indicating such a relationship in intact tissues. Ascorbic acid is known to be involved in the synthesis of collagen and reticulin, carbohydrate-containing sclero-proteins found in bones and connective tissue and in smaller amounts in other animal tissues. These frame-work proteins all presumably contain small amounts of carbohydrates and function in animals in much the same way as do the anhydrides of hexoses and pentoses in the cellulose, lignin, pentosans, etc., in plants. It has not been discovered whether ascorbic acid is concerned primarily in the activities occurring within cells or in the synthesis of essential substances outside of cell boundaries. An impressive amount of evidence has been accumulated which suggests that it is concerned in some way with determining the retention and absorption of water. Whatever its mode of action may be, there is considerable evidence that calcium is involved in its functioning.

The following review of literature and discussion represent an endeavor to correlate some of the known but hitherto widely scattered facts concerning the physiological interrelations of ascorbic acid and calcium in animals and plants and to point out suggestive similarities in their action in the two groups of organisms. The subject is discussed under the following topics:

I. Relation of calcium to the boundary structure of cells.
II. Ascorbic acid in relation to cytoplasm and cell surfaces, cell walls, and intercellular substances.
III. Some aspects of the chemical nature of the cell wall and intercellular substances.
IV. Loss of ascorbic acid in metabolic processes.
V. Similarity of some physiological effects of ascorbic acid and calcium.

I. RELATION OF CALCIUM TO THE BOUNDARY STRUCTURE OF CELLS. 1. *In animals.* The protoplasmic cell surface or plasma membrane is generally considered to be a delicate but essential structure, not many molecules in thickness and containing lipid, probably associated with protein, as a major component. Microscopic studies have revealed the existence of a readily visible

cortical layer of cytoplasm, the behavior of which has been shown by Heilbrunn and co-workers (58) to be largely controlled by calcium (see also Just (67)). These investigators observed that calcium ions have a stiffening effect upon the ectoplasm of amoeba but cause a decrease in viscosity of the endoplasm. It has also been found that calcium in the surrounding medium is not essential for the formation and maintenance of the surface layer. Chambers (23, 24) reported that Arbacia eggs immersed in a mixture of sodium and potassium chloride in proportions of 19:1, in the concentrations found in sea water, and at a pH of 7.0 will divide for many generations, the blastomeres separating as individual cells because of lack of intercellular cement material. He concluded that an addition of surface material from the underlying cytoplasm must occur. If the surface layer is injured at some point, the cytoplasm flows out and disintegration of the surface layer spreads out over the entire cell. However, repair of an injury, which exposes the internal cytoplasm, is impossible in the absence of calcium because of inability of the protoplasm to form a new interface. In the presence of a low concentration of calcium, partial coagulation around the point of injury occurs and a new protoplasmic surface layer develops at the boundary between the healthy cytoplasm and the coagulating surface. The latter is supposed to afford a solid substrate against which surface substances may accumulate. The repair of the protoplasmic surface thus depends upon a previous formation of an extraneous coat.

As cells become older the surface may become modified by secretions of various types such as mineral matter in the formation of bone. Such surface coatings are not essential to single cells but they are necessary for multicellular organisms. This is true, for example, of the hyaline layer in developing eggs, a material which Kopac (73) considered to function as an intercellular cement. Previous observations by Galtsoff (40) and Gray (47) had suggested that the surface layer of isolated cells is homologous to the intercellular cement of multicellular organisms. Intercellular substances form the foundation of all fibrous structures such as the bone matrix, cartilage, dentin, the vascular endothelium, and of all non-epithelial cement substances. In some tissues such as cartilage and connective tissue the volume of intercellular substances may be very large in comparison to that of the cell itself. In the absence of calcium the intercellular cement breaks down to a soluble substance, thereby becoming dispersed in the medium. Many investigators have contributed to our knowledge of calcium as a stabilizer of this structure in animal tissues (117, 118, 119, 59, 79, 47, 57, 58, 98, 25, 23, 73, 152).

Zweifach (152) found that the permeability of the capillaries is determined almost entirely by the nature of the cement, and apparently also in part by an adsorbed layer of protein, rather than by the cells themselves. He observed that lack of calcium in the perfusion fluid weakens the cement substances between the endothelial cells with the result that extravasation of both fluids and blood cells occurs. When the circulating fluid is changed to one containing calcium the cement substance is re-formed and the normal permeability is re-established. Zweifach suggests that a similar cement probably exists in other epithelial membranes such as periosteum, kidney tubules and epithelium.

There is comparatively little known concerning the ratio of calcium in the interior of the cell to that in surface membranes and surrounding fluids. Scott (130) found that practically all of the calcium in the nerve fibers is located in the nerve sheath. He concludes that since only a small portion of the sheath contains aqueous channels, the local concentration may be ten to twenty times that which would be expected if the calcium were uniformly distributed in the nerve fiber. The distribution of calcium between the cells and extracellular fluids of skeletal muscles and liver in dogs was studied by Eichelberger, McLean and Catterall (32). They applied accepted methods for calculating the distribution of sodium and potassium to similar calculations for the distribution of calcium and magnesium. Their results indicate that the concentration of calcium in the cells is 80 per cent lower than that in the extracellular fluid. They considered the possibility that all the calcium in the tissues may be extracellular and concluded that unless some of it is actually within the cells, a part of it must be in a unionized combination with some extracellular substance other than the protein of the extracellular fluid, possibly the connective tissue fibers. They did not suggest, however, the possibility of a linkage of a portion of the calcium with a constituent of an intercellular cement substance to form an unreadily soluble compound.

There is considerable evidence which shows that the stability of intercellular substances is attained only when bases in addition to calcium are present in the surrounding medium (99). The generally accepted view is that magnesium, potassium and sodium all enter into the final equilibrium in the animal cell and that one cannot completely compensate for the absence of another (47, 48).

2. *In plants.* The plasma membrane of the plant cell as in the animal is supported by a "gel" layer of cytoplasm (126) but there is still insufficient evidence to support the assumption that calcium is essential to its formation. Comparatively small amounts of substances are found between the cells in plants. The relationship of the middle lamella to the surrounding cells is clearly shown and it is distinctly a double layer deposited by the two adjoining cell surfaces (1). In a limited sense the wall of the plant cell, especially its outer older portion, is comparable to the intercellular substances in animal tissues. By special staining it can, in fact, be shown that at least in some types of tissues, the intercellular material between adjoining cells is, like the middle lamella in plant tissues, really a double layer.

Reed (109) found that nuclear division occurs in spirogyra in the absence of calcium but cell division often is not completed, because of lack of formation of the middle lamella, which is known to consist chiefly of calcium pectate (105, 90, 91, 52, and others). True and co-workers (140, 141) have made important contributions to our knowledge of the rôle of calcium in cell walls. Eckerson, working in co-operation with these investigators, employed microchemical methods (141) in a study of seedlings grown in solutions of potassium salts similar to those from which True and Bartlett (142) had previously found a leaching of ions. She observed "(1) that ions readily entered the cells of the roots, (2) that within twenty-four hours calcium ions began to diffuse out of the calcium pectate of the middle lamella, (3) potassium pectate was formed instead

of the calcium salt and this substance being readily soluble in water soon dissolved, (4) at this stage, sugars, amino acids and salts, chiefly magnesium, diffused rapidly out of the roots." In similar tests with magnesium solutions it was found that magnesium pectate replaced calcium pectate in the cell walls. These studies showed that the action of potassium and magnesium in the surrounding solution upon the calcium pectate of the middle lamella not only changed the character of the wall but also altered greatly the permeability of the protoplasmic membrane next to it. That calcium is necessary also for the normal development and functioning of the cytoplasm is recognized (141, 136, 11). It should be emphasized, however, that the evidence presented above indicates clearly that calcium has a function in the nutrient medium aside from its uses within the cells.

In conclusion, it may be stated that calcium functions in the extraneous coats of cells in both plant and animal organisms. In its absence, these coverings are dissolved, the constituent substances becoming dispersed in the surrounding medium. There is considerable evidence also which indicates that calcium is essential for the production and maintenance of the cytoplasm. It increases the viscosity of the cytoplasm in the cortical region and decreases that in the interior of the cell.

II. ASCORBIC ACID IN RELATION TO CYTOPLASM AND CELL SURFACES, CELL WALLS AND INTERCELLULAR SUBSTANCES. 1. *In animals.* One group of investigators hold that ascorbic acid is essential for the production of the intercellular matrix, a structure consisting of collagen or a related substance as a basic constituent whereas another group maintains that its primary influence is upon the functioning of cells. The former group (3, 151, and others) claim that if ascorbic acid is unavailable a liquid product which lacks the ability to gel is formed in the intercellular areas. In a study of scorbutic tissues, Wolbach (150) observed vacuolation of the fibroblasts, usually at their extremities, and held that the intercellular liquid very probably has its source in the cytoplasmic vacuoles. He thought that the vacuoles may be considered as evidence of degeneration (cytoplasmic presumably) or the consequence of secretion of an abnormal product. He accepted the latter interpretation. Dalldorf (30) who also holds this view describes the action of ascorbic acid as follows:

> Under certain conditions the type cell, the fibroblast, lies in an amorphous ground substance within which fibrils (reticulum) are formed which may in turn become gathered into wavy bands of collagen. In this transformation the fibrils seem to become cemented together by a translucent matrix, the formation suggesting a colloid phenomenon, the setting of a gel. It is precisely this phase of the formation of intercellular materials which may be completely controlled by vitamin C. Thus in guinea pigs which have been depleted of vitamin C, the ground substance and fibroblasts are present as in health but fibrils or collagen are not formed. When the deficiency is satisfied, translucent bundles or masses of collagenous materials reappear within eighteen hours. The formation of intercellular material of bone (osteoid tissue) and of teeth (dentin) may be similarly controlled by withholding or supplying vitamin C.

Höjer (64) advanced the hypothesis that vitamin C (ascorbic acid) is necessary for the proper functioning of cells in general and for specialized cells in particular.

Fish and Harris (38) and Ham and Elliott (50) also accepted the view that vitamin C is somehow concerned in the metabolic processes of cells. The latter investigators held the evidence for the gelation theory to be entirely inadequate. MacLean, Sheppard and McHenry (84) also obtained no evidence in support of the gelation theory. They showed that ascorbic acid deficiency causes a failure in those special cells which are concerned in the formation of calcific tissue, as in bones and teeth and the intercellular cement. Morphological changes in the blood vessel walls have not been observed in ascorbic acid-deficient animals but presumably the intercellular cement breaks down as it does in the absence of calcium. Meyer (96) presented histological evidence of effects of lack of ascorbic acid upon the structure of the cells themselves. He observed a widely distributed depletion and destruction of the cytoplasm and cell membranes in guinea pigs kept on a scurvy-producing diet. Vacuolation was common in many organs. Most of the other workers in this field have not observed a degenerating condition of the cytoplasm and cell membranes but its occurrence should not be surprising in a disease which, in severe form, causes such general tissue breakdown. The lack of agreement as to the range of tissues which may show histological defects in scurvy is probably in part a consequence of differences in the severity of the disease in the animals examined.

In recovery from scorbutus, the collagen deposit is said by Wolbach (150) to be restricted to the near vicinity of the fibroblasts, the fibrils tending to follow the cell outlines. He described the first material with the staining properties of collagen as homogeneous, resembling lightly-stained amyloid and closely applied to the cytoplasm of the cell itself and in some instances to fibrils extending beyond the apparent outlines of the cells. He noted that it was also present about cells when no fibrils were observed. Hansen (51) and Mall (87, 88) held that collagen fibrils originate as a direct modification of the surface cytoplasm of the fibroblast and its processes. Lewis (77) presented evidence supporting this view. She observed fibrils in an early stage as slightly more refractive lines within the cytoplasm of the cell which later formed slender fibers grouped in bundles outside of the cell. Other investigators (6, 7, 8, 54) have described collagen fibrils as forming in collagen independently of the immediate proximity of cells. It is possible that certain phases of collagen synthesis occur within the boundary of the cell but that completion of the product is intercellular. Hence, although histological evidence may suggest that the action of ascorbic acid is primarily intercellular, to designate it as such without regard to the less readily observable intracellular phases seems unjustifiable.

If ascorbic acid really is concerned in extra-cellular processes its concentration at cell surfaces might be expected. There is some evidence from cytological studies, which, though inconclusive, because the reaction employed is not specific for ascorbic acid, nevertheless shows that a silver nitrate-reducing substance is present in the cytoplasm and intercellular spaces (20, 21, 147). Bourne called attention to the possibility that different lipoids may be concerned at times with the silver reaction in the adrenals.

Evidence of an effect of ascorbic acid upon surface activity of colloidal solu-

tions is shown in work reported by Keller and Künzel (69), "surface activity" being understood as the force which causes dropping fluids to assume a spherical form, the smaller the drops the higher the activity. All substances which alter the surface tension of a solution medium, such as water, are said to be surface active. The investigators found that solutions of thiamine (vitamin B_1), riboflavin (vitamin B_2) and ascorbic acid do not show surface activity in themselves, but that under certain conditions they can influence this type of behavior in lecithin-cholesterol mixtures. The greatest activity was observed in the higher dilutions and also under the closest approach to physiological conditions with respect to pH. It was pointed out that the physical effects of vitamins may be very important and that their mode of operation cannot be explained by chemical structure and affinity alone.

2. *In plants.* Reid (112) made a study of some of the physical and chemical changes occurring in the growing region of the primary root of cowpea seedlings. The following regions were examined: embryonic, intermediate to embryonic

TABLE 1

Relative values per cell in cells of different ages

REGION	GREEN WEIGHT	DRY WEIGHT	WATER	NITROGEN	PHOSPHORUS	SURFACE AREA	TOTAL ASCORBIC ACID	TOTAL ASCORBIC ACID/TOTAL SURFACE AREA
Embryonic.....	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Intermediate.....	2.8	2.0	3.5	2.0	1.8	2.3	2.6	1.1
Elongation.....	13.7	4.8	16.9	4.0	4.2	7.3	7.9	1.1
Intermediate.....	32.0	8.0	43.2	5.8	5.3	11.8	11.8	1.0
Maturation.....	32.0	7.9	46.3	5.5	4.5		11.3	

and elongation, elongation, intermediate to elongation and maturation, and maturation. Continuous increases in volume, water, dry matter, nitrogen, and phosphorus per cell occurred during the transition from the embryonic to the mature condition. The results of these studies (112, 113) expressed in terms of relative values per cell (the average value for a cell in the embryonic region being taken as 1) are summarized in table 1.

It may be observed that the increase in surface of expanding cells parallels the increase in ascorbic acid and that surface area-increase is the only one of the various factors studied which shows a parallel relationship with increase in ascorbic acid. Phosphorus gained relatively as rapidly as nitrogen and possibly slightly more rapidly during the time of most active expansion and it was suggested that an accumulation of a phospholipin such as lecithin or a closely related compound may be involved in this increase. Bessey, Menten and King (18) reported a close relationship between ascorbic acid and complex lipids in animal tissues. In a study of the distribution of ascorbic acid in animal tissues, Bourne (21) pointed out that cells with a high concentration of substances such as lipochrome pigments, lipoids and fats, cholesterol and its esters contain large

quantities of ascorbic acid. The increase in phosphorus during the embryonic and early growth phases of the root cells above mentioned and the decrease during maturation are presumably related chiefly to changes in the content of nucleoproteins and nucleic acids. The relatively high content of ascorbic acid per unit of dry matter in the region of expansion was an outstanding feature of the observations. The dry substance in the region of elongation contained 60 per cent more ascorbic acid than that of the embryonic region where nuclear activity is high and 30 per cent more than the region of maturation where cellulose accumulation appears to be the dominant activity. The data suggest that the increase in ascorbic acid is associated with an increase in the cytoplasm or some of its constituents.

Further investigations of similar nature with other types of plants are necessary before drawing conclusions as to the significance of the results cited above. The quantitative relations with surface area here found do suggest, however, that ascorbic acid may tend to be concentrated at and near cell surfaces in metabolically active cells and that it may consequently have important functions in connection with cell expansion, development of the wall, and absorptive and secretory processes. A discussion of suggested relations of ascorbic acid to these activities will be presented in section V. Additional support for the hypothesis that ascorbic acid functions at cell surfaces is afforded by evidence suggesting that the surfaces of plant cells contain substances with an acid reaction (102, 103). Howe (66) found that the pH values of root hairs range from 6.0 to 6.8 in the plants tested, but she did not state whether the acidity could be ascribed in part to the presence of pectic materials known to be important constituents of root hair walls (120). In investigations made with a wide variety of plant materials the writer has observed that ascorbic acid leaches out readily from most of them when coarsely comminuted in metaphosphoric acid and the mixture allowed to stand for a half-hour or longer. These observations suggest that the ascorbic acid in the tissues, with the possible exception of that in the embryonic cells, moves readily from cell to cell through or on the surfaces of the cell walls. Another type of observation which is of interest in connection with a cell-surface relation of ascorbic acid in absorption was made in tests with highly illuminated, actively growing cowpea plants (Reid, unpublished data), the roots of which were immersed in black-walled jars containing a dilute solution of indophenol (1:125,000). A reduction of the dye equivalent to a milligram of ascorbic acid per plant occurred within a period of two hours. This reducing activity was equal to about one-ninth of the total reducing activity in the minced and extracted tissues of similar plants with all organs included. In other tests, following decolorization of the solution, additional dye was added, this procedure being repeated throughout the day. The total quantity of dye then reduced in the solution was equal to approximately one-half of the calculated total indophenol-reducing activity within the plant. The substance oxidized by the dye was apparently again reduced in the tissues, as determinations of the reducing activity of plants kept in the indophenol solution showed that no appreciable diminution had occurred during the period of immersion of

the roots. When plants in the bleached dye solutions were subsequently kept in darkness for several hours, a partial return of color in the solution was noted, but a change to red color indicated a more acid condition of the medium. A marked turbidity of the external solution signifying a leaching of substances from the roots was observed following the dye treatment, the leaching being also noticeable after a period in darkness. These tests should be repeated and other tests made with plants kept in darkness during the period of immersion of the roots in the dye. Preliminary studies have shown little reducing activity. It thus appears that the action of the dye on the roots occurs chiefly as a consequence of exposure of the tops to light. The nature of the reaction of the dye with the root cells is not clear, but, because of its rapidity and the rather large size of the dye molecules, it may presumably be related to the existence of a readily oxidizable substance in the membrane (58, 93; see also 22). Further experiments with the dye solution adjusted at different pH values should give somewhat more definite clues as to whether or not ascorbic acid is primarily involved in the reaction.

Schreiner and Sullivan (128, 129) studied the reducing action of the roots of wheat seedlings on solutions of sodium selenite and found that it was stimulated by a faintly acid reaction and by light and was most marked intracellularly in the parenchyma cells of the root tip. They considered it probable that the reduction is due to the metabolic activities of the roots, the reaction involving some unstable non-enzymatic substance comparable to the oxyorganic acids, or to complex, unsaturated compounds comparable to dextrose and levulose or unsaturated fatty acids. (It is now known that ascorbic acid will reduce solutions of sodium selenite.) Heffter (56) concluded that a type of reduction process, apparently similar to that studied later by Schreiner and Sullivan, is caused by the labile hydrogen of the sulphydryl group of certain proteins. It should be possible by further investigation, using selenite solutions with different hydrogen ion concentrations, to determine whether the type of reduction in intact roots herein described may be concerned primarily with ascorbic acid or with sulphydryl compounds.

III. Some aspects of the chemical nature of cell membranes, cell walls and intercellular substances. 1. *In animals.* The physical state of intercellular materials has been studied by a number of investigators and as a result of their observations the viewpoint has developed that "cells repose in a jelly, not upon a water bed" (27, 28, 17, 85; see also, Editorial, The Lancet (74)). On the other hand there is comparatively little definite knowledge concerning the chemical nature of the intercellular substance in animal cells. Gray (47) stated that in the connective tissue of young vertebrates it appears to consist of mucoid (a glyco-protein known to contain sugars and uronic acids) with fibrils of collagen and elastin. Halliburton (49), Chittenden and Gies (26), and Van Lier (143), also have presented evidence of the occurrence of mucoids between the fibrils of connective tissue. Data obtained by Bensley (17) in a study of the reaction of paramoecia in bullae of subcutaneous connective tissue suggested the presence in the intercellular areas of a viscid substance, possibly acid in reac-

tion, digestible by pancreatin but not by pepsin, and with staining properties resembling those of mucins. The fact that this substance could be extracted with lime water also suggested that it may be mucoid. Zweifach (152) found that the intercellular cement substance of the endothelium of the blood vessels behaves as though it were a calcium salt, probably a proteinate, and that it is digestible by trypsin. The possibility of a carbohydrate linkage with the protein was not suggested. Ferry (37) studied the jelly layer of Arenicola eggs and concluded that it is essentially a polysaccharide.

A possible relation between the water-holding capacity of tissues and their content of certain types of carbohydrate-protein complexes is suggested in studies conducted by Heringa and Weidinger (61). They measured the amount of water taken up by the cornea and sclera after they were in equilibrium with water vapor of a certain partial pressure and found that in water vapor tension above 0.9 (90 per cent) the sclera fails to take up additional water whereas the cornea continues to swell up to about 1000 per cent of its dry material. They interpret this result as seeming to indicate that there is inserted between the connective tissue fibrils a substance, probably mucoid, which differs in cornea and sclera. Their hypothesis is based upon the fact that the cornea was found to contain 20 per cent of mucoid and the sclera 13 per cent and that after the extraction of the mucoid the cornea lost the greater part of its swelling capacity. Using the Sorensen-Haugaard adaptation of the Orcin reaction, Grassman and Schleich (46) studied the carbohydrate content of animal skin and found that it contained unimolecular proportions of glucose and galactose. From this finding they concluded that the carbohydrate was present in the form of lactose. They also attempted to determine the significance of the substance surrounding the collagen fibers in a study of collagen fibers from cow's skin. They consider that their results afford good reason for the assumption that the lactose is a constituent of collagen and not of the chemically undefined interfibrillar substance. However, their evidence that the interfibrillar substance does not also contain small amounts of carbohydrates is not entirely convincing. Schneider (127) also has shown that collagen contains a small amount of firmly bound sugar. Beek (15) found that the carbohydrates from collagen were not fermented by galactose-active yeast and concluded that neither d-glucose nor d-galactose forms a considerable part of the sugars. He expressed the opinion that the carbohydrates may be *l*-isomers of the two sugars and noted that Bell and Baldwin (16) had found *l*-galactose as a component of a polysaccharide of animal origin.

Bensley (17) suggested that the cement substance of collagenic fibers may be a modified form of the ground substance and that the chemical and physical differences between reticular and collagenic tissue may be due to this difference in the relation of the fibers to the ground substance. She says that "the difference in reactions of reticular and collagenic fibers with dilute acetic acid may be explained on the basis of this relation. Undoubtedly the ground substance is in a complex colloidal state. It is well known that weak acids increase the capacity of lyophyllic colloids to absorb water. The hydration of the ground

or cement substance due to the acetic acid may render its refractive index the same as that of the fibrillae of the collagenic fiber and thereby make them both invisible. That reticular fibers do not react in this way may be explained by the fact that the ground substance enveloping them is not in the nature of a cement substance, i.e., there is not so much of it and it is not so condensed. Therefore, its hydration capacity is not increased to such a great extent and the refractive index is not appreciably changed." Observations made by Heringa and Weidinger (62) suggest that differences in chemical composition of reticulin and collagen are, in part at least, responsible for the differences in behavior. They also found that reticulin does not swell appreciably in acids and that it has a higher sulphur content than collagen. The sulphur, chiefly present as cystine, is in side chains, and by tending to diminish the side chain distances tends to prevent the intermicellar penetration of water. Thus in a very early stage of development a non-swelling substance, reticulin, is contained in skin, thereby enabling it to exist in a watery medium. Later, this substance changes to collagen which allows water to pass. It seems probable that during maturation of tissues changes may occur also in the ground substance as suggested by Bensley. Further study of this aspect of the problem appears necessary.

Northrup and Kunitz (101) accounted for the high viscosity of gelatin and its variation with pH by the existence of two forms:—one form occurring as insoluble micellae and the other a soluble form distributed between the micellae and the outer solution. They do not say whether or not it may be possible that differences in solubility of fibrillar and interfibrillar substances are involved in producing these results.

Höjer (64) reported finding a collagen atrophy in the cartilage in scurvy, especially marked in the columns of the proliferating zone and in their neighborhood. He observed that sections stained with methylene blue did not differ from the normal in regard to chondroitin-sulphuric acid. It would appear from this observation that if ascorbic acid is used directly in the production of a protein-carbohydrate complex it must be in relation to some special type which presumably is used in comparatively small amounts such as in the intercellular cement of the epithelial cells of certain tissues.

2. *In plants.* The chemical nature of the outer wall layer or middle lamella of young expanding cells is not known definitely. In the early stages it is generally considered to be chiefly pectic material such as protopectin or pectin which later changes into insoluble compounds mainly as the calcium salt of some pectic substance. There is lack of agreement as to the exact form of the latter though many workers have considered it to be calcium pectate, a salt of pectic acid. It is not known whether or not these substances are linked to protein or more probably to phosphoprotein molecules in the early stages of cell surface formation. After and also during the later phases of cell expansion, the pectic material is generally supposed to combine with calcium to form the insoluble compound above-mentioned. Pectic acid is known to be a complex molecule composed of sugars such as glucose or galactose and in some cases pentoses, together with uronic acids, chiefly galacturonic and glycuronic. When ascorbic acid is heated

with hydrochloric acid, a carbon atom is detached and the remaining compound gives the furfural reaction characteristic of pentoses.

Farr and Eckerson (36) observed that cells of higher plants (see also, Barrows (12)) are surrounded by wall-layers composed of cellulose particles embedded in an isotropic matrix, which was shown by later work (53, 35) to have some of the characteristics of pectic material. Wergin (146) studied the structure of cotton fibers and reported also that the fibrils consist of cellulose particles, considerably smaller than the units described by Farr and Eckerson. Wieler (149) investigated the cell-wall structure in a large number of plants including that of cotton fibers and he, too, observed cellulose particles not very different in size from those described by Farr and Eckerson. Concerning the nature of the cementing material surrounding the cellulose particles Farr (35) states that there seems to be no reason for considering it as anything other than a surface coating left by the cytoplasm upon each separate particle and between the successive layers of fibrils which form the wall lamellae. (Chambers (24) has pointed out that in animal cells the outer hyaline layer is formed as a secretory product left behind by the receding protoplasm.) It is to be inferred from the general trend of evidence in the studies of Farr and her associates that the cytoplasm, perhaps especially the cortical layers, contains appreciable amounts of pectic materials. Compton (29) has shown that the high hygroscopic moisture content of cotton fibers at various stages of development is due chiefly to the presence of water-soluble carbohydrates and pectic material.

There is some indication of chemical similarity in the cementing material which binds together the cellulose layers in the plant cell wall and the collagen and elastin fibrils in the intercellular matrix of animal tissues, namely, the presence of complex carbohydrates containing sugars and in many cases also of uronic acids. If present in both kinds of cells the function of these substances is likely to be somewhat similar in plants and in animals and they may play highly important rôles as hydrating and geling agents. The results of Heringa and Weidinger (61, 62) previously mentioned strongly suggest the importance of these complexes in determining the water-binding capacity of the connective tissues in animals.

Ruskin (123) has shown that ascorbic acid combines readily with calcium and Ruskin and Jonnard (124) that it combines with protein but much more readily if calcium also is present. The possibility of a relation of ascorbic acid to the pectic-like substance which may be involved in gelation processes in plant and animal tissues brings to attention a crucial problem. Available experimental evidence thus indicates that ascorbic acid may be qualified to act as a carrier of calcium in embryonic tissues but it is possible also that it helps to prevent premature calcification, and that it may be utilized directly (though possibly only in plants) in the synthesis of some special carbohydrate fraction. The quantitative relations which have been found between ascorbic acid and surface area in expanding root tip cells suggest that it may be a constituent of the middle lamella, the primary cell wall and the cytoplasm. The indophenol-reducing activity of the ascorbic acid in the wall may be preserved during the phases of

expansion where the potassium and possibly magnesium to calcium ratio is presumably considerably higher and the phosphorus to nitrogen ratio slightly higher (112, 113) than later when maturation processes become dominant.

Allen (1) expressed the view that the middle lamella is not merely an intercellular substance of cement but that it may function as a "plastic region in the growing cell wall which is in a measure adaptable to the changing form and size of the protoplast itself, and to the firm resistant layers whose form must correspond to that of the protoplast at the time of their deposition." He also said that "the evidence seems to indicate that the pectic layer continues to increase in thickness about as long as the cambial cell is increasing in size. It is possible that the attainment by the cell of its adult size marks the limit of growth of the middle lamella. In this case we might say that pectic acid is deposited so long as the metabolic processes of the cell result in a plus which is expressed in cell growth, but that later, when a metabolic equilibrium has been established, or when the excess of food is stored instead of being used for growth, or when the protoplast degenerates, a predominance of other cell wall materials is deposited. The evidence for such a view, however, is far from complete."

The relation of ascorbic acid to growth of the cell wall was studied in the root-cell investigations previously mentioned (113). It was observed that ascorbic acid increased approximately so long as the cell continued to expand. During maturation a decrease was observed, becoming more marked as full development was attained. Ascorbic acid has also been found (Reid, unpublished data) as a constituent of young cotton fibers. Older, still growing fibers also contain it but in a lower concentration on a green weight basis. The content per cell at successive stages of development was not determined. Pectic substances have also been found in cotton fibers (53) and relatively more in young than in old fibers (148, 29).

Comparisons of differences in composition of cementing material of the walls in fungi and higher plants are of special interest with respect to possible interrelations of ascorbic acid and calcium in cell-wall development. Fungi are said to contain little, if any, ascorbic acid and calcium is generally considered as unessential or, possibly, as essential only in very slight traces. It is known, however, that growth of some fungi, such as yeast, is much more rapid if calcium is present in the nutrient medium. It is possible also that biological tests for determining the antiscorbutic value of fungi may be difficult to conduct without danger of loss of ascorbic acid before the supplement is consumed. Hence, small amounts of the vitamin would not be easy to evaluate by this method. In the higher plants both ascorbic acid and calcium are present and doubtless both are essential. Types of higher plants lacking chlorophyll require calcium but much less than chlorophyllous types, and they also contain much less ascorbic acid than the latter plants (43). Chlorophyllous tissues such as leaves contain much higher concentrations of both ascorbic acid and calcium than the non-green parts, with the possible exception of certain regions of extremely high metabolic activity as in the growing regions of roots. The reason why calcium and ascorbic acid may be essential in appreciable amounts for non-green plants but

not for fungi is still obscure. It should be possible to determine whether or not differences in composition of the walls are chiefly involved. Pectic substances have been reported in the cell walls of cellulose-forming fungi (36) but the cement materials surrounding and binding the cellulose layers undoubtedly differ somewhat in chemical composition from those in higher plants. Presumably the pectic substances are not present as calcium salts since, as previously stated, only slight traces of calcium, if any, are essential for fungi. Calcium is found, however, in the walls of non-chlorophyllous higher plants. The general trend of evidence as to the distribution of ascorbic acid and calcium in different groups of plants definitely suggests the possibility of a mutual relationship to calcium pectate or to the calcium salt of some related pectic substance occurring in the middle lamella. Further study is necessary before drawing definite conclusions as to a direct relation of this vitamin to cell-wall development.

The materials surrounding the cells of plant and animal tissues have been herein shown to consist of solid units bound together by a cement substance. Carbohydrates or carbohydrate-containing complexes are important constituents of these binding materials, probably influencing strongly their hydrating and gelling properties. There is evidence that the cement substance in both types of tissue is a calcium salt, in the plant a salt of pectic acid, possibly linked to protein in embryonic cells and in the animal probably a proteinate, though it may be one with a carbohydrate complex.

IV. Loss of ascorbic acid in metabolic processes. 1. *In plants.* Losses amounting to as much as 15 to 20 per cent in the absolute amount of ascorbic acid in young cowpea plants have been found to occur at night, but apparently only at temperatures which are favorable to growth (110, 111, 114, 115). Other investigators have observed the same phenomenon in other types of plants (97, 133, 71). The writer has observed similar losses also in older growing cowpea plants but, because of the larger and somewhat more variable sizes of the plants, the losses are somewhat difficult to determine quantitatively. Gains in the absolute amount of ascorbic acid during periods of darkness do occur so long as the stored food reserves in the cotyledons last. Following this period losses at night are found (114). The evidence suggests that only seedlings and possibly sprouting tubers and bulbs with special types of storage reserves have the capacity to synthesize ascorbic acid during periods of darkness. It appears that plants at other stages of growth either lack the ability to convert some of their carbohydrate reserves into ascorbic acid or synthesis occurs but is not measurable because the losses are considerably greater than the gains.

More recent work (116) affords evidence which supports the latter concept and definitely indicates an ability of plant tissues to synthesize ascorbic acid during periods of darkness. Tests were made with excised tomato roots (sixtieth explants) grown in a modified Pfeffer solution containing one per cent sucrose, thiamin (vitamin B_1), and pyridoxin (vitamin B_6) but no added ascorbic acid. A definite indophenol-reducing action of the root extracts was observed with approximately the same speed of reaction as occurs with ascorbic acid and the reaction was of such magnitude as to eliminate the possibility of transference

of the total quantity from the original explants. From these results it seems fairly definite that excised tomato roots in sterile cultures have the capacity to utilize sucrose in the synthesis of ascorbic acid. It is also probable that roots of intact plants have the ability to form ascorbic acid at night by utilizing some of the stored carbohydrates but that the newly synthesized portion is not measurable because the losses are greater than the gains. The nightly losses may thus be appreciably greater than the decreases as observed in the assays. Presumably even greater losses occur in the daytime than at night but they are difficult to determine because the rate of synthesis tends to be greater than that of loss. However, in rapidly growing plants with low carbohydrate reserves the writer has obtained indications of possible losses in the afternoon on cloudy days. The results thus suggest that ascorbic acid may play a much more important rôle in the economy of the plant than has heretofore been realized. The fate of the disappearing ascorbic acid is as yet undetermined but, as previously suggested, it may possibly be used for a constructive purpose such as in the synthesis of some special constituent of the pectic substances in the growing regions. Support for this assumption is to be found in the apparent loss in total ascorbic acid in root cells during maturation (113) and also by the fact that at favorable temperatures a very rapid growth of roots usually occurs in young cowpea plants from the eighth to the tenth days, at which time there is a lag in the general upward trend of the curve representing the absolute amount of ascorbic acid in the plant (114). Kohman and Porter (72) have recently reported that in tomato plants with roots severed the losses in ascorbic acid at night are less than in intact plants.

The structure of the ascorbic acid molecule is such that it could presumably be converted into the uronic acid of d-gulose, but a compound of this type has not been reported as occurring in plant tissues. On the other hand it is possible that the loss of ascorbic acid which occurs in darkness may be, at least in part, a result of oxidation and indirectly a consequence of a lowering of the sugar content. It has been shown in *in vitro* experiments by Munilla and Vogelsinger (100) that sugars protect ascorbic acid from oxidation. A lowering of the sugar content might thus affect not only the synthesis of ascorbic acid but also its maintenance. Results of other tests have shown that in green plants exposed to light, their ascorbic acid may be protected from oxidation by the chlorophyll (108). Possible losses of ascorbic acid by oxidation are difficult to determine, at least in some types of tissue, because of the difficulty of complete removal of the hydrogen sulfide employed in reducing the dehydro form of ascorbic acid and also because a number of substances combine with hydrogen sulfide to form compounds which react like ascorbic acid (135). Moldtmann (97) makes the unqualified statement, however, that the losses of ascorbic acid at night are not a result of oxidation. The need of further study of this problem is evident.

Mention should also be made of the fact that plants grown in solutions containing high concentrations of basic ions (Ca, Mg or K) even when the nutrient medium is maintained at a favorable pH, have definitely, though, except in the case of magnesium, not markedly, lower ascorbic acid values than plants grown

in solutions containing medium or low concentrations of these ions. (104, 10, Reid, unpublished data.) It is not known whether these decreases signify lessened synthesis or increased utilization. If, during the growth process, the vitamin undergoes a modification with eventual combination with basic ions, the latter hypothesis is more probably the correct one.

2. *In animals.* Considerably less ascorbic acid is excreted than is ingested by the human body but no satisfactory answer is available as to where or how the loss occurs. Smith (134) in a review and discussion of the question of human needs of ascorbic acid, summarizes as follows the daily requirement in terms of milligrams per kilogram of body weight: "Infants from 3 to 8 mg., children from 6.4 to 7.5 mg., and adults from 0.7 to 1.6 mg." The data are held to suggest that ascorbic acid requirements are related both to body weight and to metabolic rate, the effect of the latter being the more pronounced. None of the investigators who have studied the relation between intake and excretion of ascorbic acid have suggested the possibility of utilization of the disappearing portion for a directly constructive purpose. It should be pointed out that the period of maximum requirement of calcium is coincident with the period of most rapid growth and most rapid disappearance of ascorbic acid.

There is considerable evidence that the utilization of calcium by the tissues is influenced by ascorbic acid (139, 64, 125, 34, 4, 82, 83, 75, 76, 84, 131). Daniels et al. (31), however, reported finding no relation between ascorbic acid ingestion of between 2.7 and 12.5 mgm. per kilogram and calcium retention in children, and Henry and Kon (60) found no effect of an addition of 2 mgm. daily of ascorbic acid on the retention of calcium in rats kept on a diet low in calcium. Mallon and Lord (89) observed that addition daily of 5 cc. of lemon juice to young growing rats supplied with a diet containing 0.166 per cent calcium caused no increase or decrease in calcium retention. It seems possible, however, that ascorbic acid might influence calcium retention with a diet high in calcium. Hawley, Frazier, Button and Stevens (55) studied the effect of acid and alkali upon the amount of ascorbic acid excreted in the urine of human subjects. They found a pronounced decrease in excretion when the urinary pH was in the alkaline range of 7.5 to 8.1 and the effect was apparently not due to reversible oxidation. Tislowitz (138) reported that the injection of large doses of ascorbic acid into dogs caused a ten per cent increase in the alkali reserve. The results of other investigations suggest a partial explanation of these observations. Robertson, Ropes and Bauer (121) studied the degradation of mucins and polysaccharides by ascorbic acid and hydrogen peroxide. They found that the system acted on starch, pectin, flaxseed mucilage, the polysaccharides of synovial fluids and cartilage and the capsules of various types of pneumococci but caused no apparent change in ovo-mucin, agar-agar or gelatin. Later work (122) by these investigators showed that the viscosity of gelatin also was reduced by this treatment. The question arises as to whether the breakdown of the macromolecules may have been influenced to some extent by the removal of traces of calcium and possibly of other bases from the reacting materials. Grant (45) presented evidence which shows that mucus, a glycoprotein, is an immediate

source of calcium in gastric secretions and that it constitutes an important factor in the reduction of gastric acidity. The presence of varying amounts of mucus in secretions of different types is held by this investigator to be responsible for calcium and acidity variations in gastric secretions. The work of Oden (102) suggests that the carbohydrate moiety of the mucin molecule may well be involved in these pH regulatory reactions. He obtained evidence that pectic substances may act as regulators of the H and OH ion content of circulating solutions in plant tissues in so far as these come into contact with the pectic materials. That pectic substances may exert some as yet unidentified beneficial influence in the digestive tract in addition to their action as lubricants is generally recognized (70, 144, 145). Werch et al. (145) suggest that an effect of pectin on the pH of the intestinal contents is the most probable explanation of the beneficial action of pectin in the treatment of diarrheas caused by some types of intestinal pathogens.

Losses of ascorbic acid are herein shown to occur in both plants and animals. Metabolic losses occur during growth in some, if not in all plants, and in animals, the losses are much greater during rapid growth than in the adult stage. It is suggested that ascorbic acid may be used in the synthesis of some substance essential in growing regions. There is considerable evidence that the utilization of calcium in the tissues is influenced by ascorbic acid and conversely, the utilization of ascorbic acid by the tissues presumably is influenced by calcium.

V. Similarity in some physiological effects of ascorbic acid and calcium. 1. *In animals.* a. *Growth.* The necessity of ascorbic acid for growth of higher animals is generally recognized but direct experimental evidence of such a relationship is somewhat limited. Young animals succumb to its absence in the diet so suddenly that a retarding effect on growth is often not observable. Anderson and Smith (2) in paired feeding experiments with guinea pigs found that animals given scurvy-preventing supplements and whose food consumption was iso-caloric with that of scorbutic animals attained a greater weight. A relationship of ascorbic acid to growth of cells is suggested by results reported by Glick and Biskind (44). They observed that the size and ascorbic acid content of the cells of the adrenal cortex increased regularly from the fetal to the adult stage of the animal. The necessity of calcium for growth has long been recognized. Boelter and Greenberg (19) have recently presented further proof that growth is retarded when the supply of calcium is low.

b. *Water relations.* Calcium and ascorbic acid are probably jointly involved in exerting an effect upon the water content of cells and tissues. The extremely rapid losses in weight often observed in guinea pigs with severe scurvy could presumably be a consequence of an upset water balance associated with a disturbed equilibrium between the solid and liquid cytoplasmic phases resulting in lowered ability of the cells to retain water. It would be of interest to find whether or not at a stage preceding the final collapse there was not increased hydration of the cell colloids as a consequence of release of calcium from the intercellular cement. McHenry, Reedman and Sheppard (95) and Sheppard and McHenry (132) found in feeding tests with guinea pigs that lack of ascorbic

acid caused a diminished retention of water which largely accounted for the observed differences in body weight. They concluded that ascorbic acid is apparently concerned directly or indirectly with water retention. Ley (78) described a condition in very young infants, characterized by a disturbance in water metabolism and marked loss in weight which responded quickly to administration of ascorbic acid.

It is possible that the therapeutic value of ascorbic acid in fever-producing infections may be a consequence of a beneficial influence upon the water balance.[1] Balcar, Sansum and Woodyatt (9) suggested that "fevers are due to a deficit of free water resulting in an abnormal tendency on the part of the colloids of the body to bind water." They held that the poison of the disease leads to changes in the cell colloids, increasing their hydration capacities, so that they take up and bind more water. They suggested that beneficial results would follow if enough water were introduced to saturate the increased affinities of the colloids and provide excess free water. There is considerable evidence that ascorbic acid may reduce the water-binding capacity of certain types of proteins. Pozzi (107) has shown that it decreases the viscosity of gelatin solutions and McClean and Hale (94) and Madinaveitia and Quibell (86) have reported similar effects on mucin preparations from vitreous humor. The work of Robertson, Ropes and Bauer (121, 122) previously mentioned has shown that the reduction in viscosity on treatment of gelatin, synovial fluid, gastric and salivary mucins with ascorbic acid and hydrogen sulfide causes a reduction in viscosity which is irreversible, due to a breakdown of macromolecules. Honaga (65) reported that in most diseases causing fever the blood calcium is lessened at the height of the fever whereas with blood potassium the opposite occurs. During convalescence normal values are restored. He found also that induced fever in the rabbit increases the calcium content of the liver and muscles and decreases that of the skin. The increase in calcium in some tissues during fever may possibly be principally intracellular and the loss from others may be chiefly from extracellular sources. The increase in calcium content of certain tissues suggests that the calcium may be directly involved in a hydrating and gelling action upon the cell colloids. The favorable action of ascorbic acid may be a consequence of release of the bound water and removal of the excess intracellular calcium.

c. *Permeability.* Chambers (24) described three types of cellular membranes in multicellular organisms, in the first of which the intercellular cement is present in such an appreciable amount that the permeability of the membrane is determined by it rather than by the cells. The endothelial lining of the blood vessels is an example of this type. Permeability of the second type of membrane is determined by both the intercellular cement and the cells, the intestinal mucosa being an example of this type. In the third kind of membrane the cells fit together so closely that the cement has a negligible influence on permeability. An example of this type is to be found in the walls of the proximal tubules of the kidneys. It has been previously shown in this discussion that the intercellular

[1] This suggestion was made to the writer by the late Dr. Lillias D. Frances.

cement, at least that of certain epithelial tissues, is a calcium salt, the formation and, presumably, also the maintenance of which is dependent upon the presence of ascorbic acid. It seems probable that membranes of the first type should be affected most severely by shortage of essential cement materials. If either calcium or ascorbic acid is lacking, extravasation of both blood cells and fluids occurs especially if the tissue is subjected to extra strain or pressure. When both calcium and ascorbic acid are deficient, earlier and more pronounced histological defects would presumably be expected. Excessive intake of either ascorbic acid or calcium with an inadequate supply of the other would possibly be harmful. Because of the apparently interrelated action of calcium and ascorbic acid it might be expected also that a deficiency of either of these substances would cause some of the symptoms characteristic of lack of the other. This, in fact, does occur. The results obtained by Boelter and Greenberg (19) have shown that shortage of calcium causes retardation of growth, reduced activity, marked weakening of the muscles and paralysis of the hind legs, high basal metabolic rate, internal hemorrhages, and osteoporosis, all of which are characteristic scurvy symptoms.

d. *Detoxifying action.* There is considerable evidence that ascorbic acid protects the body against the toxic effects of certain chemically unrelated substances such as lead, benzene, arsenicals, some types of amines, and barbiturates but the basis for a chemical action has not been found. It is possible that physical-chemical factors which may influence surface action may be chiefly concerned. If this is true, and if the influence of ascorbic acid is exerted principally at or near cell surfaces, as herein suggested, it becomes unnecessary to postulate specific chemical reactions with these substances to explain the detoxifying effect.[2] The suggestion has also been made that the detoxifying action of ascorbic acid against certain chemicals may be related to a tightening effect upon either or both the plasma membrane and the cement substances in the cell walls. Such a change in either of these structures would tend to retard the passage of large molecules.[3] Undoubtedly calcium would be involved in this and in some of the other detoxifying reactions but present knowledge is insufficient to warrant making a positive statement regarding such an influence. The beneficial action of ascorbic acid in alleviating the toxic effects of lead can probably be explained as a precipitating action on the lead. It should be added here that if the detoxification of some types of substances is a surface reaction, synthesis of ascorbic acid may be a surface phenomenon also, since the two processes appear to be related according to evidence presented by Longenecker, Fricke and King (81).

e. *Muscle action.* Friedman (39) studied the effect of ascorbic acid deficiency on smooth muscle responsiveness to stimulation with histamine and found a greatly reduced reaction as compared to that in normal tissues. Basu and Biswas (13) and Basu and Ray (14) studied the influence of ascorbic acid on different types of muscles and found that it augments contraction, brings about

[2] This suggestion was made by Mr. Chas. H. Binkley.

[3] This suggestion was made by Dr. L. E. Yocum.

quicker relaxation and delays the onset of fatigue. Their experiments showed also that the effects are not due to any alterations of pH. Results also of tests reported by Gantenbein (41), Matthes (92), and Linstead and Krayer (80) indicate a stimulating effect of calcium on muscle action. Lack of calcium also affects the muscles causing loss of tone and flaccid paralysis. Basu and Biswas pointed out that the response to ascorbic acid is somewhat similar to that to calcium (Gellhorn, 42). The present concept of muscle action is said to be that "calcium is released on excitation from its combination with protein and this released calcium causes reversible gelation in the interior of the protoplasm." If calcium is added to the external fluid, the gelated condition is induced much more readily and to a greater extent than before, and consequently the response is greater. The suggestion was made that since ascorbic acid is known to cause gelation in different fluids, it is probable that it also causes gelation in muscle plasm. The suggestion could also be made that ascorbic acid aids in the recovery process and influences intracellularly the solation phase of the reaction.

The possibility of interrelated effects of ascorbic acid and calcium on other tissues in which surface activities play an outstandingly important rôle such as those of the nervous system, glandular organs and white blood corpuscles has not been investigated with this particular aspect in view.

f. *Effect of ascorbic acid and calcium on catheptic enzymes.* Additional reason for postulating an interacting relationship in the functioning of calcium and ascorbic acid is to be found in the fact that both of these substances have an activating effect on catheptic proteolytic enzymes (68, 33, 5).

2. *In plants.* Direct proof of the growth relations of ascorbic acid in higher plants is difficult. The same is true also of a possible connection of this vitamin with other physiological functions because it is always present in metabolically active tissues. If, as some of the reported observations suggest, ascorbic acid functions in the cytoplasm and at surfaces of cells, as has been previously shown for calcium, it may presumably exert an important influence upon the intake and retention of water and also of mineral nutrients. It has been shown (137, 63) that absorption of mineral salts by roots occurs chiefly during active growth or in roots with the potentiality for active growth. It has also been demonstrated (106) that the maximum penetration of potassium nitrate into soybean roots occurs between 1 and 4 mm. from the tip. The penetration of salts was found to diminish progressively toward the older region of the root and to stop almost completely at 10 mm. Osterhaut (103) observed that ammonia entered the fresh surface of Valonia cytoplasm squeezed out of cells and stated that it did so by forming a compound with an acid at the surface. The available evidence indicates that the region of active growth and absorption in roots is identical with that having the highest ascorbic acid concentration per unit area of cell surface. The general trend of evidence strongly suggests the possibility of the movement of water and mineral nutrients through the cementing material of the walls in the growing region of the root in much the same fashion as materials are transferred through the cement substance surrounding the endothelial cells of the blood vessels in animals.

SUMMARY

Calcium gives stability to cell surfaces and intercellular substances of both plant and animal tissues and there are indications that it may act in somewhat similar manner in the two groups.

Ascorbic acid is necessary for the production of certain types of intercellular materials, and possibly also for the production and maintenance of cytoplasm and normal plasma membranes in animals and there is considerable suggestive evidence that it may perform a similar function in plants. Whether its primary action is to influence the functioning of cells or the production of intercellular colloids or whether it is involved in both of these types of activity has not been fully determined.

Similarities are shown in the physical constitution of the cell walls of plants and the intercellular substances of animal connective tissues, namely, that they both contain solid units, different as to chemical nature, and bound together by cement substances containing units which are chemically somewhat similar. It is supposed that in plants the latter materials may aid in the maintenance of a plastic state during expansion and later, on setting firmly, impart strength to the structure. The plastic state presumably is maintained by retarding precipitation of calcium whereas the maturation phase of the process presumably allows freedom in calcium deposition. There is suggestive evidence that a somewhat similar sequence of changes in form of the intercellular substances occurs also in some types of animal tissues.

Collagen, which is concerned in the gelling action of the intercellular matrix in animals, contains sugars. The mucoid-like ground substance surrounding the collagen fibrils is held by some investigators to contain both sugars and uronic acids. Sugars and uronic acids are present in plant tissues as constituents of the pectic materials, complex carbohydrates with marked hydration and gelling properties. Supposedly calcium plays a rôle in the gelling action of these sugar-uronic acid complexes.

Ascorbic acid exhibits properties which may adapt it for functioning at cell surfaces; namely, it shows definite surface activity in lecithin and cholesterol solutions, and it combines readily with protein in the presence of calcium. Presumably it could act as a carrier of calcium in embryonic regions, it could also be involved in preventing premature calcification, and its structure is such that it could probably itself be changed to form a special component of the pectic acid molecule.

The increase in surface of expanding cowpea root tips at successive stages of development approximately parallels the increase in ascorbic acid. During maturation the values become lower which is interpreted as possibly indicating a transformation of the vitamin to a non-reducing substance during maturation processes. It is suggested that ascorbic acid may be used in the formation of calcium pectate or a related substance. Further suggestion either of the utilization or the destruction of ascorbic acid in plants is supplied by the fact that, under conditions suitable for growth, marked losses of the vitamin occur in plants kept in darkness and presumably also in those kept in light. There are

unaccountable losses of ascorbic acid from animals also, though the magnitude and manner of loss are difficult to determine.

In addition to the parallelism between ascorbic acid and cell surface increases in growing root cells, further suggestive evidence that vitamin C may be concentrated at cell surfaces is as follows: Cell surfaces of plant tissues contain substances with an acid reaction, a rapid reduction of indophenol occurs when roots of plants with tops exposed to light are immersed in the dye solution, and in animal tissues there is considerable tendency for deposition of silver granules to occur at cell boundaries and in intercellular spaces as well as in the cytoplasm when the tissues are placed in acidified silver nitrate solution.

The very low requirements or possibly unessential nature of ascorbic acid and calcium in the development of fungi and the essentiality of calcium and probable essentiality of ascorbic acid in higher green plants is pointed out as is also the fact that calcium pectate is a constituent of the walls of higher plants, even of non-chlorophyllous types, but is not necessarily present in fungi.

The availability of relatively high concentrations of calcium, magnesium and potassium appears to have a favorable influence upon retention of ascorbic acid in animals as evidenced by lessened excretion. High concentrations of these elements cause lower values in plants, but it has not been determined whether the difference in content is due primarily to decreased synthesis or to increased utilization.

Glycoproteins such as mucus may serve as sources of calcium in gastric secretions and there is suggestive evidence that the carbohydrate moiety of the mucin molecule may be involved in pH regulatory reactions.

Similarities are shown in the physiological effects of calcium and ascorbic acid with respect to water relations, permeability and growth in plants and animals and also to their effect on muscular action and on catheptic enzymes in animals. Ascorbic acid exerts a detoxifying action in animals against a number of substances. There is as yet little more than suggestive evidence that calcium may also be involved in these reactions.

If ascorbic acid and calcium function co-operatively as has been shown in maintenance of the intercellular cement in the endothelium of the blood vessels, it should be expected that there would be marked similarity, although not necessarily complete identicality in symptoms when one or the other of these substances is lacking. The observed similarity in some of the deficiency symptoms of the two substances affords support for this assumption.

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